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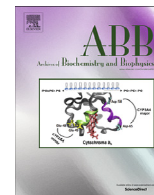

# Na,K-ATPase reconstituted in ternary liposome: The presence of cholesterol affects protein activity and thermal stability

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Differential scanning calorimetry (DSC) was applied to investigate the effect of cholesterol on the thermotropic properties of the lipid membrane (DPPC and DPPE). The thermostability and unfolding of solubilized and reconstituted Na,K-ATPase in DPPC:DPPE:cholesterol-liposomes was also studied to gain insight into the role of cholesterol in the Na,K-ATPase modulation of enzyme function and activity. The tertiary system (DPPC:DPPE:cholesterol) (molar ratio DPPC:DPPE equal 1:1) when cholesterol content was increased from 0% up to 40% results in a slight decrease in the temperature of transition and enthalpy, and an increase in width.

We observed that, without heating treatment, at 37 °C, the activity was higher for 20 mol% cholesterol. However, thermal inactivation experiments showed that the enzyme activity loss time depends on the cholesterol membrane content.

The unfolding of the enzyme incorporated to liposomes of DPPC:DPPE (1:1 mol) with different cholesterol contents, ranging from 0% to 40% mol was also studied by DSC.

Some differences between the thermograms indicate that the presence of lipids promotes a conformational change in protein structure and this change is enough to change the way Na,K-ATPase thermally unfolds.

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## Introduction

Na,K-ATPase is a member of the P-type family of active cation transport proteins. It is present in the plasma membrane of virtually all animal cells. The enzyme complex consists of two main polypeptide chains: the α-subunit and β subunit (∼110 kDa and ∼35–50 kDa, respectively) [1,2]. A third subunit is a small hydrophobic protein (γ subunit in the kidney) that is associated with the Na,K-ATPase, with a relative molecular mass of 7–12 kDa [3]. The enzyme uses energy from ATP hydrolysis to transport three $Na^+$ ions out of cells and two $K^+$ ions in against their concentration gradients [4]. The $Na^+$ gradient generated drives many transport processes through co-transporters (sodium glucose) and exchangers ($Na^+/Ca^{2+}$). This gradient also drives amino acids and vitamin transport into cells [4]. Beside this, the electrochemical gradient is essential for physiological processes such as electrical excitability, nerve transmission, and muscle contraction [5,6]. The transport of $Na^+$ and $K^+$ is accomplished by conformational changes. In general, two main states exist: the sodium-bound E1 state and the potassium-bound E2 state [7].

Crystal structures of NKA at 5 Å resolution or higher have elucidated some states of this protein. Two were analogous to $E2{\cdot}Pi{\cdot}2K^+$. The first was the crystal of the enzyme extracted from pig kidney in the $E2{\cdot}2Rb^+{\cdot}MgF_x$ state, at 3.5 Å resolution [8] and the second was the enzyme extracted from shark glands rectal $E2{\cdot}2K^+{\cdot}MgF_4^{2-}$ at 2.4 Å resolution [9].

Two other structures were linked to ouabain states: with low affinity $E2{\cdot}2K^+{\cdot}MgF_4^{2-}{\cdot}$ouabain at 2.8 Å resolution [10] and with high affinity $E2P{\cdot}nH^+{\cdot}$ouabain, at a resolution of 4.6 Å [11]. The last state was E2P·ouabain with a resolution of 3.5 Å [12]. The most recent was the $E1(AlF^{4-}){\cdot}ADP{\cdot}3Na^+$ form, analogous to the transition state ($E1{\sim}P{\cdot}ADP{\cdot}3Na^+$) [13].

The fluidity of the lipid bilayer can modulate the activity of several enzymes associated with a membrane such as Na,K-ATPase. Cholesterol is an essential lipid component of the plasma membranes of the cells of higher animals and it is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane. Although cholesterol has a number of different functions in animal cells, one of its

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primary roles is as a modulator of the physical properties and lateral organization of the plasma membrane lipid bilayer [14–16]. Changes in the cholesterol content of biological membranes are known to alter the properties of the lipid lamella and hence affect the activity of membrane-bound enzymes [17]. Lipid rafts can be defined as sphingolipid and sterol-rich lipid bilayer domains that exist in the liquid-ordered (Lo) state [18]. Rafts and related membrane microdomains such as caveolae have been suggested to play important roles in sorting of membrane molecules and in signal transduction in animal cells [19]. The very complex lipid composition of the plasma membrane and formation of specific lipid microdomains such as rafts are almost certainly very important in the regulation of Na,K-ATPase [20].

Differential scanning calorimetry (DSC)[1] is known to be one of the most powerful methods for studying protein unfolding, as it provides information on the structural organization and interactions of cooperative domains in proteins [21].

Moreover, this technique is frequently used for determining changes in model lipid membranes like liposomes and provides accurate information, quickly and easily, about both physical and energetic properties of a material [22].

A recent work from our group showed a quantitative description of how some factors such as ions, substrate and lipid influence the stability of NKA using DSC. A recovery of the stability could be verified when solubilized Na,K-ATPase was reconstituted in liposome vesicles of DPPC and DPPE, indicating the crucial role of lipids in the stabilization of the protein structure [23].

Here, we studied the effect of lipid composition in NKA stability. For that purpose, different ratios of cholesterol were added in the lipid microenvironment of NKA reconstituted in DPPC:DPPE systems and how the different ratios of cholesterol affect the ATPase activity of this enzyme was also verified.

## Material and methods

### Material

All solutions were prepared with Millipore Direct-Q ultra-pure apyrogenic water. All the reagents were of the highest purity commercially available. Trichloroacetic acid (TCA), tris[hydroxymethyl]aminomethane (Tris), N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES), adenosine 5′-triphosphate tris salt (ATP), bovine serum albumin (BSA), dodecyloctaethyleneglycol ($C_{12}E_8$), cholesterol, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma. Ethylenediaminetetracetic acid (EDTA), potassium chloride, sodium chloride and magnesium chloride were obtained from Merck. Biobeads was acquired from BioRad.

### Preparation of Na,K-ATPase

Solubilized Na,K-ATPase was obtained from dark red medulla of rabbit kidney as previously described in [24].

### Preparation of the liposome and proteoliposome

Liposomes were prepared by the extrusion method as described below. The lipids were dissolved in chloroform:methanol (2:1) and dried with nitrogen flow, forming a lipid film, and then dried overnight under vacuum for complete solvent removal. The film was suspended in 5 mM Tris–HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl, by incubation for 1 h, at 70 °C and stirred using a vortex at 10 min intervals. The mixture was then extruded (11 times) through a 100 nm polycarbonate membrane under hot air flow.

Proteoliposomes of DPPC:DPPE (1:1 w/w) and cholesterol at different mol% (0, 10, 20, 30, and 40 mol%) were prepared by the co-solubilization method using a 1:3 (w/w) lipid:protein ratio in the absence of cholesterol and 1:6 (w/w) in the presence of cholesterol, as previously described in [25,26].

The determination of liposomes and proteoliposomes size distribution was performed by Dynamic Light Scattering (DLS), by using a N5 Submicron Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA, USA).

### Analysis of the protein

Protein concentration was estimated in the presence of 2% (w/w) SDS as described in [24]. Determination of the protein in the proteoliposome was performed according to the methodology described in [16]. Bovine serum albumin was used as standard.

### Enzymatic activity measurements

Activity of the enzyme ATPase was assayed discontinuously at 37 °C in a final volume of 1.0 mL by phosphate release quantification as described in [27]. The reaction was initiated by the addition of the enzyme, and it was stopped with 0.5 mL of cold 30% TCA. Samples were centrifuged at 4000*g* prior to determination of phosphate. Standard assay conditions were 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM $MgCl_2$, and 50 mM NaCl. Assays were performed in triplicate. Controls without added enzyme were included in each experiment to quantify non-enzymatic hydrolysis of substrate. The initial rates of hydrolysis were constant for at least 30 min, provided that less than 5% of substrate was consumed in the reaction.

Thermal inactivation studies of reconstituted Na,K-ATPase preparations were performed in thermostatic water baths at temperatures ranging from 50.0 to 62.5 °C. After 1–250 min, the residual enzymatic activity in 50 μL aliquots were assayed at 37.0 °C, as previously described. The inactivation constant and the thermodynamic parameters were calculated as described in [28].

### Differential scanning calorimetry

Transition temperatures ($T_m$) of the liposome, the proteoliposome, and the solubilized protein were measured by Differential Scanning Calorimetry (DSC). Samples and reference (buffer) were placed in the calorimeter and analyzed by using a Nano-DSC II from Calorimetry Sciences Corporation, CSC (Lindon, Utah, USA). All samples were degassed under vacuum (140 mbar) for 30 min prior to use, and scanned from 20 to 100 °C at an average heating and cooling rate of 0.5 °C/min, under 3 atm pressure. The baseline was determined by filling both sample and reference cells with buffer. Data analysis was performed with the aid of the fitting program Cpcalc provided by CSC. The plot and deconvolution were carried out by using MicroCal Origin 8.0 version (Gaussian deconvolutions with $R^2 \geqslant 0.996$).

## Results and discussion

The solubilized and purified Na,K-ATPase was reconstituted into lipid vesicles mimicking the natural membranes. In our study, the use of cholesterol in a proteoliposome preparation resulted in

[1] *Abbreviations used:* DSC, differential scanning calorimetry; TCA, trichloroacetic acid; BSA, bovine serum albumin; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; EDTA, ethylenediaminetetracetic acid.

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