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Addition of subunit γ, K^+ ions, and lipid restores the thermal stability of solubilized Na,K-ATPase

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ABSTRACT

Differential scanning calorimetry (DSC) was applied to ascertain the effect caused by K^+, Na^+, ATP, detergent, DPPC, DPPE, and subunit γ on the thermostability of Na,K-ATPase. The enthalpy variation (ΔH) for the thermal denaturation of the membrane-bound is twice the ΔH value obtained for solubilized Na,K-ATPase. Denaturation occurs in five steps for membrane-bound against three steps for the solubilized enzyme, therefore a multi-step unfolding process. In the presence of Na^+, the melting temperature is 61.6 °C, and the ΔH is lower as compared with the ΔH obtained in the presence or in the absence of K^+. Addition of ATP does not alter the transition temperatures significantly, but the shape of the curve is modified. Subunit γ probably stabilizes Na,K-ATPase in the beginning of thermal unfolding, and different amounts of detergents in the solubilized sample change the protein stability. Reconstitution of Na,K-ATPase into a liposome shows that lipids exert a protector effect. These results reveal differences on the thermostability depending on the conformation of Na,K-ATPase. They are relevant because it allows a comparison with future studies, e.g. how the composition of the membrane interferes on the stability of Na, K-ATPase, elucidating the importance of the lipid type contained in cell membrane.

Introduction

The discovery of Na,K-ATPase by Skou [1] was a critical step in the study of the cell as a basic unit of animal life [2]. The Na,K-pump moves three Na^+ ions out of and two K^+ ions into the cell for each hydrolyzed ATP molecule. The sodium pump is ubiquitous because it is necessary for regulation of the volume of animal cells. Moreover, the Na^+ gradient is the energy source for facilitated transport of other ions and metabolites. The generation and maintenance of membrane potentials are necessary for nerve transmission and muscle contraction and excitability [3–5]. A number of studies have suggested that Na,K-ATPase interacts with neighboring membrane proteins in caveolae and organizes cytosolic cascades of signaling proteins, so that messages can be sent to intracellular organelles in different tissues [6]. The Na,K-pump acts as receptor for the cardiac glycosides that are effective in heart failure through inhibition of the enzymatic activity [7]. Na,K-ATPase is essential for cell viability, and it has been indirectly implicated in the etiology of diseases such as hypertension and diabetes [8]. The functional Na,K-ATPase is a heterodimer, and subunits α and β are crucial to the catalytic and transport functions. In some tissues, like the kidneys, Na,K-ATPase contains a third subunit, γ, which displays a regulatory function [9]. Subunit α has a transmembrane domain formed by 10 segments: α M1–α M10. Subunit β contains a large glycosylated extracellular portion and is anchored to the membrane by a single helical segment [5]. Subunit γ or protein FXYD2 displays a single trans-membrane segment with an extracellular N terminus and a cytoplasmic C terminus [10]. New information on the structure and function of Na,K-ATPase has been obtained from crystal structures. The structure of the protein from the shark was determined at a resolution of 2.4 Å. It is similar to that of the pig protein, previously determined at 3.5 Å. It completes the structure of the ectodomain of β- subunit and identifies cholesterol at a proposed lipid-binding site. The crystal form of the shark Na,K-ATPase was also soaked with cardiotonic steroid ouabain, which provided insights into a low-affinity cardiotonic steroid-binding site in Na,K-ATPase among helices αM1, αM2 and αM4-M6 [5,11–13]. Although the crystal structure of Na,K-ATPase has been established, many studies are still required for deeper understanding of the biological pump operation [8].

Calorimetric techniques have played a pivotal role in the development of our understanding of the energetic and thermodynamic mechanisms underlying protein folding–unfolding transitions [14]. Differential Scanning Calorimetry (DSC) studies on the thermal denaturation of proteins have had a central part in the development of the current views about the factors that determine protein stability [15].

Our group has investigated the structure–function and stability of Na,K-ATPase by means of biophysical techniques such as Circu-

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lar Dichroism (CD), Infrared Absorption (IR), Spectroscopy Fluorescence Emission, Surface Tension, Dilatational Elasticity, and Small-Angle X-ray Scattering (SAXS). CD showed that the surfactant/enzyme ratio affects the aggregation of the solubilized protein with $C_{12}E_8$ [16–21]. At high detergent concentrations (greater than 2.7 mg/mL), the activity is almost completely lost, and the form $(\alpha\beta)_n$ does not exist, because the detergent leads to dissociation of subunits α and β. SAXS revealed that subunits α self-assemble into α_4 after dissociation [21]. The IR and fluorescence emission studies on the enzyme reconstituted into liposome evidenced that the cytoplasmic domain of subunit α is responsible for aggregation of the enzyme in a proteoliposome sample [20]. Here we have used calorimetry for direct measurement of the thermal stability, so as to confirm previous results and increase our understanding of how detergent influences protein stability. DSC is a powerful technique to assess how factors such as the presence of ions (Na^+ and K^+), substrate (ATP), lipids, and subunit γ alter protein conformation and change its thermal stability.

Material and methods

Material

All the solutions were prepared with Millipore Direct-Q ultrapure apyrogenic water. All the reagents were of the highest commercially available purity. Tris[hydroxymethyl]aminomethane (Tris), N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES), adenosine 5′-Triphosphate tris salt (ATP), bovine serum albumin (BSA), dodecyloctaethyleneglycol ($C_{12}E_8$), dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma. Ethylenediaminetetracetic acid (EDTA), ammonium bicarbonate (NH_4HCO_3), potassium chloride (KCl), sodium chloride (NaCl), and trichloroacetic acid (TCA) were obtained from Merck. Biobeads was acquired from BioRad.

Preparation of Na,K-ATPase

Membrane-bound and solubilized/purified Na,K-ATPase were obtained from the dark red medulla of the rabbit kidney as previously described in [16]. The membrane-bound enzyme was attained in homogenization buffer (20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM EDTA, and 6 mM Tris). After solubilization, the purified protein was maintained in solubilization buffer (5 mM Tris–HCl buffer, pH 7.0, containing 15 mM KCl, 6 mM EDTA and 0.005 mg/mL $C_{12}E_8$).

Extraction of subunit γ

Extraction of subunit γ was carried out as described in [22] with some modifications. Membrane-bound Na,K-ATPase (1.0 mL, 1.0 mg) was diluted 16-fold with 46% (v/v) methanol, 46% (v/v) chloroform, and 8% (v/v) 750 mM NH_4HCO_3 mixture. The sample was centrifuged for 5 min at 600*g*, and the supernatant (subunit γ) was dried under N_2 flux. The dry material was suspended in 500. Next, selective precipitation with diethyl-ether was accomplished as described in [22]. The precipitate was suspended in 500 μL of the homogenization buffer.

Preparation of the proteoliposome

A DPPC:DPPE (1:1 w/w) proteoliposome was prepared by the co-solubilization method using a lipid:protein ratio 1:3 (w/w), as previously described in [17,20].

Proteolysis with trypsin

Membrane fractions containing Na,K-ATPase were treated with trypsin at a protein:trypsin ratio 3:1 (w/w). After 30 min at 37 °C, the smaller polypeptide units and trypsin were eliminated by ultracentrifugation for 1 h, at 100,000*g* and 4 °C. The pellet was suspended in homogenization buffer.

Analysis of the protein

Concentration of the protein was estimated in the presence of 2% (w/w) SDS as described in [16]. Determination of the protein in the proteoliposome was performed according to the methodology described in [23]. Bovine serum albumin was used as standard.

Enzymatic activity

Activity of the enzyme ATPase was discontinuously assayed at 37 °C in a final volume of 1.0 mL by quantification of phosphate release as described in [24]. The reaction was initiated by addition of the enzyme, and it was stopped with 0.5 mL of cold 30% TCA solution.

Differential scanning calorimetry (DSC)

Melting temperatures (T_m) and variation of the thermal denaturation enthalpy (ΔH) of the membrane-bound Na,K-ATPase, the solubilized enzyme and the proteoliposome were measured by DSC. The samples and reference (buffer) were placed in the calorimeter and analyzed on the apparatus Nano-DSC II from Calorimetry Sciences Corporation, CSC (Lindon, Utah, USA). All the samples were degassed under vacuum (140 mbar) for 30 min before use. Scans were recorded from 20 to 90 °C at an average heating rate of 0.5 °C/min, under pressure of 3 atm. The baseline was determined by filling the sample and the reference cells with buffer solution. Data was analyzed with the fitting program CpCalc provided by CSC. The plot and deconvolution were carried out by using Origin version 8.0 (Gausian deconvolutions with $R^2 > 0.993$).

Results and discussion

To study the effects of factors such as the presence of detergent, lipids, K^+ and Na^+ ions, substrate and, subunit γ on the stability of Na,K-ATPase, we conducted DSC experiments using Na,K-ATPase from the rabbit kidney in different conditions.

The heat capacity profile of a macromolecular system undergoing a temperature-induced transition; e.g. protein unfolding is characterized by the presence of one or more peaks in the transition region. The change in enthalpy associated with the unfolding, ΔH, is the area under the curve [14]. A recent review described DSC as a tool for protein folding and stability [25].

Membrane-bound Na,K-ATPase

Fig. 1-A shows the heat capacity profile of the membrane-bound Na,K-ATPase from the rabbit kidney. Deconvolution of the endotherm indicates a five-step thermal denaturation, with transition temperatures (Tt) of 47.9, 52.9, 57.7, 62.9 and 69.0 °C (Tm ≈ 58 °C, Table 1). Grinberg et at. [26] described transition temperatures of 47.5, 54.3 and 58.4 °C (Tm ≈ 53 °C) for the membrane-bound Na,K-ATPase extracted from the pig kidney. Fodor et al. [27] found transition temperatures of 33.2, 41.5, 45.1 and 50.2 °C (Tm ≈ 44 °C) for membranous Na,K-ATPase from the shark and 49.0, 52.7, 54.6, 62.7 and 70.1 °C (Tm ≈ 54 °C) for membranous Na,K-ATPase from pig kidney. Rescan of the sample reveals that