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Biochimica et Biophysica Acta 1778 (2008) 835–843
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Stabilization of Na,K–ATPase by ionic interactions

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Received 2 May 2007; received in revised form 26 September 2007; accepted 10 December 2007
Available online 15 December 2007

Abstract

The effect of ions on the thermostability and unfolding of Na,K–ATPase from shark salt gland was studied and compared with that of Na,K–ATPase from pig kidney by using differential scanning calorimetry (DSC) and activity assays. In 1 mM histidine at pH 7, the shark enzyme inactivates rapidly at 20 °C, as does the kidney enzyme at 42 °C (but not at 20 °C). Increasing ionic strength by addition of 20 mM histidine, or of 1 mM NaCl or KCl, protects both enzymes against this rapid inactivation. As detected by DSC, the shark enzyme undergoes thermal unfolding at lower temperature ($T_m \approx 45$ °C) than does the kidney enzyme ($T_m \approx 55$ °C). Both calorimetric endotherms indicate multi-step unfolding, probably associated with different cooperative domains. Whereas the overall heat of unfolding is similar for the kidney enzyme in either 1 mM or 20 mM histidine, components with high mid-point temperatures are lost from the unfolding transition of the shark enzyme in 1 mM histidine, relative to that in 20 mM histidine. This is attributed to partial unfolding of the enzyme due to a high hydrostatic pressure during centrifugation of DSC samples at low ionic strength, which correlates with inactivation measurements. Addition of 10 mM NaCl to shark enzyme in 1 mM histidine protects against inactivation during centrifugation of the DSC sample, but incubation for 1 h at 20 °C prior to addition of NaCl results in loss of components with lower mid-point temperatures within the unfolding transition. Cations at millimolar concentration therefore afford at least two distinct modes of stabilization, likely affecting separate cooperative domains. The different thermal stabilities and denaturation temperatures of the two Na,K–ATPases correlate with the respective physiological temperatures, and may be attributed to the different lipid environments.

Keywords: Na,K–ATPase; Denaturation; Pig kidney; Shark salt gland; Differential scanning calorimetry; Electrostatic screening

1. Introduction

Na,K–ATPase, an extensively studied member of the P-type ATPase family, is involved in regulation of cell volume, development of membrane potential, and transport of nutrients and water in animal tissues [1]. It is an oligomeric complex, where the α-subunit carries out ion transport coupled to ATP hydrolysis, the β-subunit is mostly a chaperone responsible for correct membrane insertion, and the γ-subunit (an FXYD-protein) modulates transport activities of the enzyme by affecting the apparent affinities for cations [2]. The three-dimensional organization of Na,K–ATPase at atomic resolution is not yet known, and for this reason the structure of the homologous enzyme, Ca–ATPase (SERCA 1a), is widely used for interpretation of results on the former [3]. This is justified because the sequence of the Na,K–ATPase α-subunit is highly homologous to that of the Ca–ATPase, with only minor gaps and insertions. Additionally, a 3-D model of pig kidney Na,K–ATPase αβγ protomer, based on cryo-electron microscopy of two-dimensional crystals, reveals distinct similarities between the catalytic α-subunit and the Ca–ATPase, given the predicted locations of the β- and γ-subunits in Na,K–ATPase [4].

Abbreviations: CDTA, *trans*-1,2-cyclohexylenedinitrilo-tetraacetic acid; $E_2(K)$, the protein conformation in the presence of K^+; SDS, sodium dodecyl sulphate; DSC, differential scanning calorimetry

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0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.12.006

According to current structural classifications, the catalytic subunit of a P-type ATPase consists of four well-defined domains: a membrane-bound domain, extra-membranous domains involved in phosphorylation (P) or in nucleotide binding (N), and the extra-membranous hinge domain (A). Many members of the P-type ATPase family contain additional subunits that may be considered as separate domains in the overall structure of the enzyme. For the Na,K–ATPase, the two additional subunits β and γ each have a single transmembrane span. Differential scanning calorimetry (DSC) experiments with P-type ATPases, on the other hand, have been interpreted in terms of the unfolding of a smaller number of independent cooperative domains: two for the α-protomer Ca–ATPase [5] and the αβ-protomer gastric H,K–ATPase [6], and three for the αβγ-protomer renal Na,K–ATPase [7].

The common architecture of P-type ATPases implies a common mechanism of action. Transport of ions against their electrochemical gradients is coupled to ATP hydrolysis, and this coupling occurs through a conformational change (E_1–E_2) of the enzyme molecule. Differences between these two major conformations have been characterized by various methods (see Refs. [1,8] for reviews), and recently visualized in crystal structures of the Ca–ATPase [3,9]. The most profound changes are in the relative positions of the extra-membranous domains. For renal Na,K–ATPase, the E_2(K)-form (in 20 mM K^+) is more stable towards thermal denaturation than is the E_1-form (in 20 mM Na^+) [10,11]. Induction of the E_2-conformer by 10 mM Rb^+ (a congener of K^+) similarly affords protection against inactivation and trypsinolysis on incubation at 55 °C [12,13], a temperature at which thermal unfolding would otherwise occur.

To study the effect of cations and ionic strength on the stability, and the number of independent cooperative domains in the thermal unfolding of these enzymes we have chosen two almost identical proteins that function at different temperatures. In the present study, we characterize the effects of ionic strength and low concentrations of cations on the thermostability and thermal unfolding of Na,K–ATPase from tissues of different organisms: shark salt gland and pig kidney. These animals belong to the poikilotherms and homeotherms, respectively, and therefore the Na,K–ATPases are adapted to function at different temperatures (about 8 °C for shark and 36 °C for pig). Thermal unfolding [14], and inactivation at low ionic strength, take place at lower temperatures for shark salt gland Na,K–ATPase than for the enzyme from pig kidney. Protection of both shark and pig kidney Na,K–ATPases from inactivation at low ionic strength is afforded by a variety of cations at millimolar concentration. Na^+, $Tris^+$, choline and protonated histidine are equally effective and protection appears to follow a simple Debye–Hückel dependence on ionic strength, whereas K^+ is more efficient, presumably due to induction of the E_2(K)-form. Inactivation of shark Na,K–ATPase at low ionic strength, either thermally or as a result of high-speed centrifugation [15], correlates with the pattern of multi-step thermal unfolding at higher temperatures. Centrifugation at low temperature selectively removes calorimetric components at higher temperatures within the unfolding transition, whereas incubation at increased temperature removes components at lower temperatures within the transition. Again protection is afforded by increasing the ionic strength.

2. Materials and methods

2.1. Enzyme preparation

Na,K–ATPase from the salt gland of *Squalus acanthias* was prepared according to the method of Skou and Esmann [16], omitting the saponin treatment. Na,K–ATPase from pig kidney microsomal membranes was prepared by treatment with SDS and purified by differential centrifugation [17,18]. The specific activity of both enzyme preparations was approximately 30 μmol ATP hydrolysed/mg protein per min at 37 °C [19].

2.2. Stability measurements

The purified membrane preparation (stored at 5 mg protein/mL) was diluted into buffer solution containing 1 or 20 mM histidine (neutralized to pH 7.0 with CDTA at the given temperature), with or without additional salt, to a final concentration of 0.1 mg/mL and incubated for up to 3 h at different temperatures. The cation concentration of 1 mM histidine at pH 7 is calculated to be 0.1 mM (pK_a=6.05 for histidine imidazole), which is neutralized by 0.03 mM of trivalent CDTA. Steady-state Na,K–ATPase activities were assayed at the time points shown in Figs. 1 and 2 by a 10-fold dilution of protein into an assay medium containing 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$ and 3 mM ATP, with or without 1 mM ouabain, at 20 °C. Phosphate liberation after 2–6 min was measured colorimetrically [20].

2.3. Sample preparation for differential scanning calorimetry

Purified membrane preparations were diluted with 1 or 20 mM histidine (pH 7.0 at 20 °C) to a final concentration of ca. 0.1 mg/mL. The sample was either incubated for 1 h at 20 °C and centrifuged at 4 °C for 2 h (20000 rpm in a Beckman Ti70 rotor, average g=29.400) or immediately subjected to centrifugation for 2 h at 4 °C. The pellet was resuspended in the same buffer (at a protein concentration of ca. 2.4 mg/mL) and used for DSC experiments.

2.4. Differential scanning calorimetry

DSC measurements were performed using a VP-DSC calorimeter, with 0.5-mL sample and reference cells, from MicroCal, LLC (Northampton, MA). The instrumental baseline was determined before each sample scan, by filling both sample and reference cells with the buffer used for the membrane samples, and using the same scanning parameters. Samples were scanned between 5 and 95 °C, at a rate of 1 K·min^{-1}, using the passive feedback mode of the VP-DSC instrument. Immediately after completion of the heating scan, a down-scan from 95 to 5 °C was

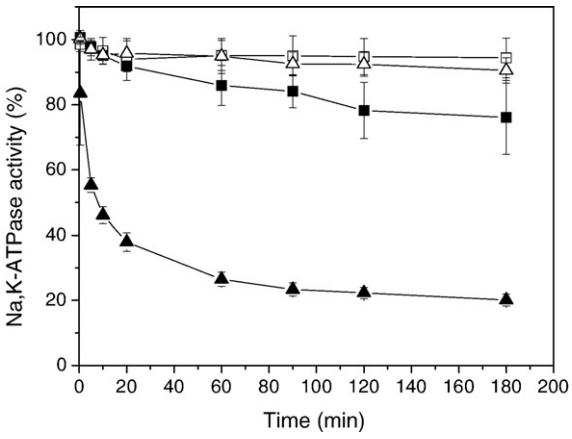

Fig. 1. Inactivation profiles for membranous Na,K–ATPase from shark salt gland. Membranes at a concentration of 0.1 mg protein/mL were incubated at different temperatures and under different ionic conditions: 1 mM histidine at 20 °C (▲);20 mM histidine at 20 °C (Δ); 1 mM histidine at 4 °C (■); and 20 mM histidine at 4 °C (□). 100% is the activity of a fully active enzyme preparation (see Methods), and averages±SD of three determinations are shown.