Cooperativity of the αβ-protomer structure in Na^+,K^+-ATPase functioning

A scanning microcalorimetry study

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Heat denaturation of the free and ligand-bound forms of purified Na^+,K^+-ATPase from pig kidney is studied with the scanning microcalorimetry technique. A single two-state transition is observed during denaturation of the free enzyme, the molar concentration of the cooperatively melting units being equal to the concentration of αβ-protomers ($M_r \approx 140\,000$). Upon interaction of the enzyme with phosphate, Mg^{2+}, and strophanthidin, but not with Na^+, the cooperativity of the protomer unfolding is lost, and the protein stabilization enthalpy becomes ≈230 kJ/mol higher. The data suggest that (i) in a functionally active enzyme form, the αβ-protomers possess a rigid structure with tight association of their subunits and domains, (ii) this structural rigidity is essential for the Na^+,K^+-ATPase functioning and (iii) there is a unique non-active conformation of the enzyme which may play an important role in its in vivo regulation.

Na^+,K^+-ATPase *αβ-Protomer* *Structure cooperativity* *Enzyme conformation* *Scanning microcalorimetry*

1. INTRODUCTION

The protein moiety of Na^+,K^+-ATPase, a membrane-bound enzyme which is responsible for transport of univalent cations across the plasma membrane of animal cells [1], consists of αβ-protomers of $M_r \approx 140000$ [2,3]. Ligand-binding and end-group analysis data suggest the enzyme molecule to be the $\alpha_2\beta_2$-diprotomer [4].

Here we report the results of a scanning microcalorimetry study revealing some novel conformational properties which seem essential for functioning of the enzyme.

2. EXPERIMENTAL

Membrane-bound Na^+,K^+-ATPase was purified from the plasma membrane fraction of pig kidney outer medulla according to Jørgensen [5] with modifications [6]. α- plus β-subunits comprised ≥95% of the protein material; the enzyme activity was ≈30 μmol P_i/mg protein per min. Protein was assayed according to the method of Lowry et al. [7] which gives correct results in the case of Na^+,K^+-ATPase [4]. Samples containing 1–2 mg protein per ml were prepared on 30 mM morpholine-cacodylate (pH 7.4) buffers whose pH value showed little dependence on temperature change ($\Delta pH/K \approx -0.006$), and equilibrated against reference solutions by dialysis.

Heat denaturation curves were registered at a scanning rate of 1 K/min in a DASM-1M microcalorimeter [8] equipped with 1 ml cells. Thermodynamic parameters were evaluated as described [8,9] and expressed per mol αβ-protomers.

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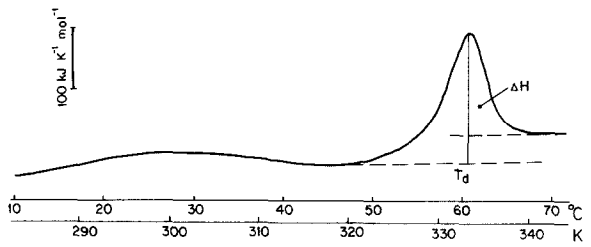

Fig.1. An experimental curve showing heat capacity of the membrane-bound Na^+,K^+-ATPase preparation as a function of temperature. Sample: Na^+,K^+-ATPase preparation suspended in 30 mM morpholine-cacodylate, pH 7.4, 10 mM morpholine-phosphate and 10 mM $MgCl_2$. Protein concentration, 1.75 ± 0.02 mg/ml.

3. RESULTS

A typical temperature dependence of heat capacity of the membrane-bound Na^+,K^+-ATPase preparation has two maxima of heat absorption, without any sign of aggregation-linked heat release (fig.1). The large high-temperature component corresponding to the protein denaturation transition [10] was subjected to computer analysis which revealed only one two-state transition in the case of the free enzyme, the molar concentration of the cooperatively melting units being equal to the concentration of $\alpha\beta$-protomers (fig.2A). An excellent fit of the experimental curve with that computed for a two-state process is a strong indication of the intrinsic reversibility of the Na^+,K^+-ATPase melting despite its failure to renature during cooling in the calorimeter cell.

Addition of the specific Na^+,K^+-ATPase ligands to the sample results in a higher protein structure stability (higher transition temperature, T_d) and in an increased denaturation enthalpy, ΔH. In the ΔH vs T_d plot the points corresponding to the examined samples fall on two parallel straight lines lying apart along the ordinate axis by 230 kJ/mol (fig.3), which is at least 3-times higher than the absolute error of the enthalpy determination. A difference in the character of the denaturation process is also evident. Unlike the free and Na^+-complexed enzyme (lower line of fig.3), the Na^+,K^+-ATPase complexes with P_i, $P_i + Mg^{2+}$ or $P_i + Mg^{2+}$ + strophanthidin (upper line) unfold in two steps via a highly populated intermediate state whose enthalpy is 550–700 kJ/mol higher than

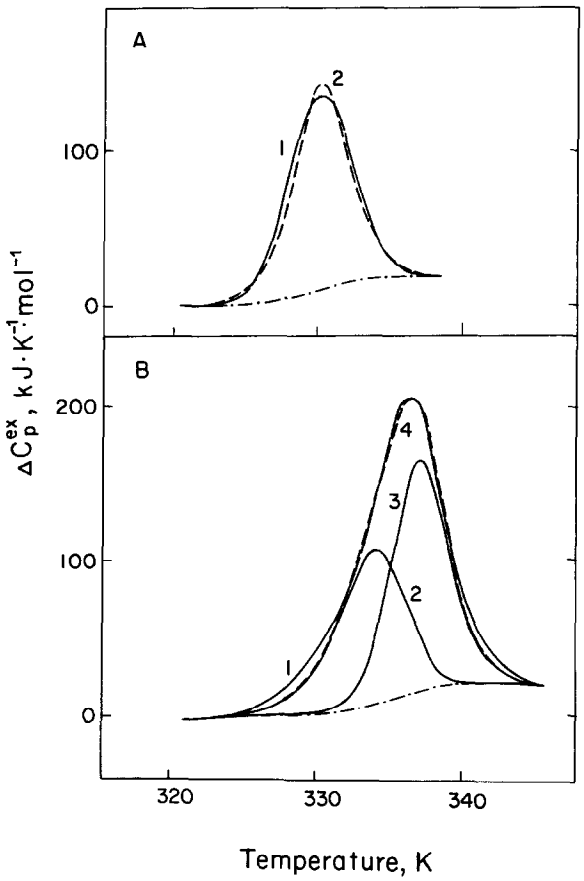

Fig.2. Deconvolution of the excess heat capacity curves to two-state transitions, made by algorithm [9] under the assumption that the molar concentration of independently melting units is equal to the concentration of $\alpha\beta$-protomers (M_r 140000). The portions of the curves are shown which correspond to the protein melting. (A) Denaturation of the free enzyme. The experimental curve (1) and its best fit by a two-state transition (2). A similar picture was observed in the presence of 100 mM NaCl. (B) Na^+,K^+-ATPase denaturation in the presence of 10 mM morpholine-phosphate, 10 mM $MgCl_2$ and 1 mM strophanthidin: experimental curve (1), individual two-state transitions (2,3) and their sum (4). Similar results were obtained when only 10 mM morpholine-phosphate with or without 10 mM $MgCl_2$ was present in the sample.

that of the initial (native) state (fig.2B). The difference is comparable with the enthalpy of the free enzyme denaturation (780 kJ/mol), so the splitting of the heat absorption peak may not be due to sample heterogeneity.

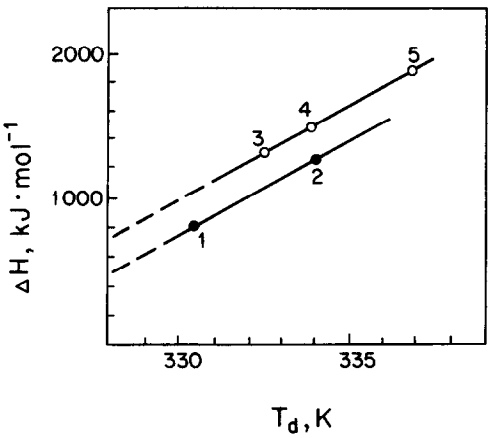

Fig.3. Denaturation enthalpy (ΔH) values replotted vs temperature of the transition midpoints (T_d). (1) Free enzyme; (2) (1) + 100 mM NaCl; (3) (1) + 10 mM morpholine-phosphate; (4) (3) + 10 mM $MgCl_2$; (5) (4) + 1 mM strophanthidin.

Thus, Na^+,K^+-ATPase can exist in two conformational states distinguishable by their stabilization enthalpy values as well as by the mechanism of heat denaturation.

4. DISCUSSION

4.1. *Properties of the Na^+,K^+-ATPase structure revealed by scanning microcalorimetry*

The results show that in the case of the ligand-free or Na^+-complexed Na^+,K^+-ATPase, representing the functionally active enzyme state, $\alpha\beta$-protomers melt independently of each other, and via a single two-state transition (fig.2A). This seems to be the first example of cooperative melting of a protein possessing a quaternary structure, and of $M_r > 20000$ (cf. [11]), and implies mechanical rigidity of the protomer structure. The independent melting of $\alpha\beta$-protomers, reflecting the lability of their association within the enzyme molecule, is in line with the observations of an easy dissociation of Na^+,K^+-ATPase into the intact protomers during formation of two-dimensional crystals or under detergent action [1]. The looseness of interaction of the rigid protomers may make the enzyme molecule capable of the $E_1 \rightleftharpoons E_2$ transitions within the functional cycle [1], for example, via the protomers' mutual shifts as proposed in [12,13].

The cooperative melting of the sample and the liability of most of the protein material to a change of its thermodynamic characteristics upon interaction with the specific Na^+,K^+-ATPase ligands (fig.2) suggests an essential homogeneity of the preparation as regards its structural and functional properties. Therefore, the 'half-of-sites' reactivity revealed in the ligand-binding experiments with purified membrane-bound Na^+,K^+-ATPase preparations [14] seems to be a property of the native enzyme rather than being due to a contamination by some inert protein material (but cf. [15,16]).

4.2. *The non-active Na^+,K^+-ATPase conformation*

It was reported in [17] that the Na^+,K^+-ATPase interaction with either P_i or Mg^{2+}, or both, results in the same isothermic heat release in each case, 175–200 kJ/mol $\alpha\beta$-protomer, while addition of K^+ has no effect. This is in agreement with our estimation of the difference between the stabilization enthalpy of the active enzyme forms and that of its complexes with P_i, Mg^{2+} and strophanthidin (fig.3). Formation of the complexes results in a loss of the cooperativity of the $\alpha\beta$-protomer unfolding (fig.2) presumably due to a loosening of the coupling between the two large parts of the protomer structure. This hypothesis is supported by the observation [18] that ouabain binding exposes at the 3/5 α-chain length a chymotrypsin-sensitive peptide bond inaccessible to protease attack in active conformations.

It follows from the results presented here and in [17] that P_i, Mg^{2+} and cardiotonic steroids, each being inhibitory for Na^+,K^+-ATPase, all induce virtually the same enzyme state which is different from both functional E_1 and E_2 conformations inducible by Na^+ and K^+, respectively [1]. A transition to this non-active conformation appears to be an intrinsic property of the enzyme molecule itself, the mechanism of the inhibitory action of each of the above substances appears to trigger this transition, and would provide for a conformational mechanism of the enzyme activity regulation.

In conclusion we wish to note that the apparent correlation of a loss of the $\alpha\beta$-protomer structure cooperativity with enzyme inactivation suggests the rigidity of the protomer to be essential for the enzyme functioning.

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