ADENOSINE-5′(*p*-FLUOROSULFONYLPHENYLPHOSPHATE) AS A TOOL FOR INVESTIGATION OF COOPERATIVE PROPERTIES OF Na,K-ATPase

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1. Introduction

Na,K-ATPase (EC 3.6.1.3.) is an oligomeric membrane-bound enzyme, whose substrate is an allosteric modifier of activity [1–3]. The enzyme has two K_m values; its affinity for Na^+ and K^+ can be controlled by ATP. The allosteric effect of ATP may be due to the changes of the cooperative interactions in the oligomeric complex of the Na-pump [4]. Here we have studied the cooperative properties of brain Na,K-ATPase by means of AFSPP, an ATP analog capable of forming an enzyme–inhibitor complex due to its covalent binding to the protein functional groups. This compound was synthesized in [5] and successfully used for the analysis of the ATP-binding sites of the proteins [5,6].

We have found that AFSPP irreversibly inhibits the activity of Na,K-ATPase and decreases (n_H) for ATP from 2.3–1.1 (at 30°C). The same influence of temperature on the n_H value for ATP was found: decrease in temperature converts the cooperativity for ATP in the Na,K-ATPase system from positive to negative. We have concluded that the type of cooperative interactions in the oligomeric complex of Na,K-ATPase is controlled by the phase state of lipids and substrate or its analogs.

Abbreviations: Na,K-ATPase, Na,K-activated, Mg-dependent adenosine triphosphate phosphohydrolase; AFSPP, adenosine-5′-(*p*-fluorosulfonylphenylphosphate); n_H, Hill coefficient; K_m, apparent Michaelis constant; ACES, *N*-(2-acetamido)-2-aminoethane sulfonic acid

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2. Materials and methods

Membrane preparations of Na,K-ATPase were obtained from the grey matter of ox brain [7]. The amounts of P_i and protein were determined by standard procedures [8,9]. The values of K_m and V for the enzymatic reaction were calculated from the curve obtained during a continuous pH-metre registration of hydrolysis of limited amounts of ATP, using the differential Michaelis-Menten equation [10–12]. The n_H values for ATP were calculated according to a classical procedure [10], using the linear portion of the curve:

$$\log \frac{v}{V-v} \quad \text{vs} \quad \log S$$

The ATPase activity was determined under optimal conditions [13] in a medium, which contained: 1–3 mM ATP, 130 mM NaCl, 20 mM KCl, 3–5 mM $MgCl_2$ and 10–30 mM imidazole (pH 7.4). The correction for Mg-ATPase, with an activity <5%, was made using 1 mM ouabain. The enzyme preincubation with AFSPP was carried out at different time intervals in the following medium: NaCl 150 mM, $MgCl_2$ 3–5 mM, ACES 3 mM, 37°C within pH 4.0–6.5. The samples were then incubated in the medium for the Na,K-ATPase activity measurement. The rate of ATP hydrolysis was constant for ⩾20 min. The constants of Na,K-ATPase inhibition by AFSPP were calculated according to [14]. Five different enzyme preparations were used in the experiments. All the experimental series were repeated at least 3 times; each experimental point is the mean of two determinations, the error ⩽2%.

3. Results and discussion

Preincubation of Na,K-ATPase with AFSPP progressively inhibits the enzyme activity. After a 100-fold dilution of the enzyme preincubated with AFSPP the inhibition is not eliminated. Thus, the effect of AFSPP is irreversible and the interaction between AFSPP and Na,K-ATPase can be described by the following reaction:

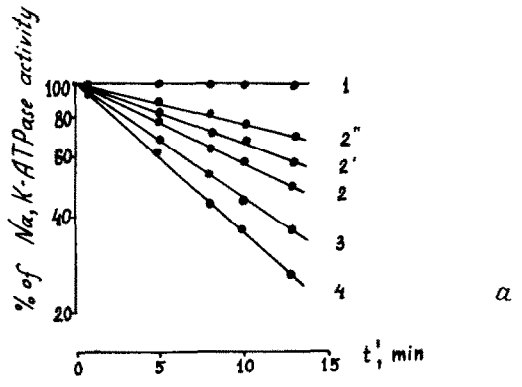
$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} E \cdot I \xrightarrow{k_{+2}} E\text{–}I$$

Fig.1a shows the time-dependence of Na,K-ATPase activity after enzyme preincubation with AFSPP. The apparent inhibition constant (k_{app}) was calculated from the formula:

$$K_{app} = \frac{0.693}{T_{1/2}}$$

where $T_{1/2}$ is the preincubation time required for 50% inactivation of ATPase at every concentration of AFSPP [14]. The values of the true inhibition constant (K_i) and those for the formation of the enzyme–inhibitor complex E–I (k_{+2}) were determined from the reciprocal plot for the k_{app} dependence versus the inhibitor concentration (fig.1b). The first step of the reaction, i.e., formation of the enzyme–inhibitor complex, is reversible and the rate of inhibition is presumably limited by this step. The K_i value equal to the k_{+1}/k_{-1} determines the inhibitor affinity for the enzyme. Here K_i = 11 mM, and $k_{+2} = 0.29\ min^{-1}$.

The presense of ATP in the preincubation medium protects the enzyme against AFSPP (fig.1a, curves 2′ and 2″); the effective concentrations of AFSPP and ATP are comparable. This may be due to the fact that both compounds compete for the ATP-binding sites



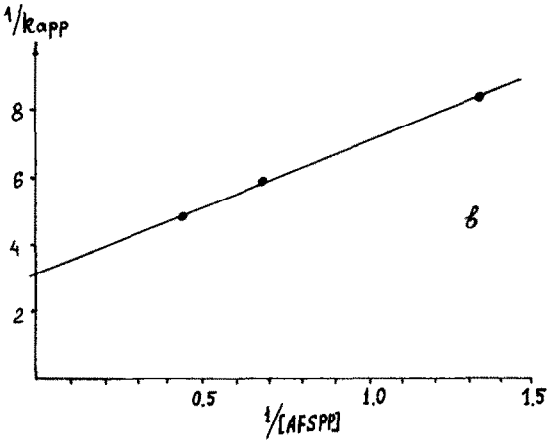


Fig.1. Inhibition of Na,K-ATPase activity by AFSPP. (a) Dependence of inhibition on time of preincubation with AFSPP (pH 6.5) in the absence of ATP (curves 1–4) and after addition of 1.5 mM (curve 2′) and 4 mM (curve 2″) ATP. [AFSPP]: none, curve 1; 0.75 mM, curves 2,2′,2″; 1.5 mM, curve 3; 2.3 mM, curve 4. After preincubation the samples were diluted 100-fold and incubated 10 min in the medium for Na,K-ATPase activity determination; then the increase in P_i was measured as in section 2. Abscissa, Na,K-ATPase activity (in log scale); ordinate, time of enzyme preincubation with AFSPP. (b) Determination of Na,K-ATPase activity inhibition constants by AFSPP. For explanation see text.

of the enzyme. The value of k_{app} depends on the pH of the medium during the enzyme preincubation with AFSPP. The group interacting with AFSPP has a pK_a value of 4.8 (fig.2). This indicates that the carboxylic group of the protein may be involved in the reaction [10].

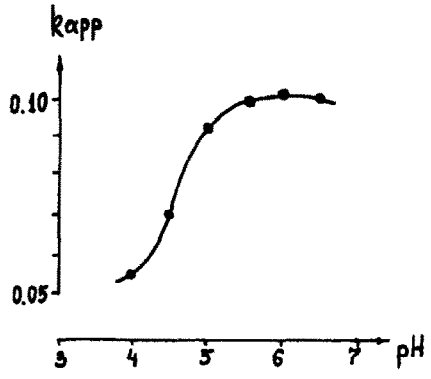


Fig.2. Dependence of the apparent constant of Na,K-ATPase inhibition by AFSPP on pH of the preincubation medium. Conditions as in fig.1a. The pH of each sample was adjusted to the required value by 2-amino, 2-methyl-1,3-propandiol.

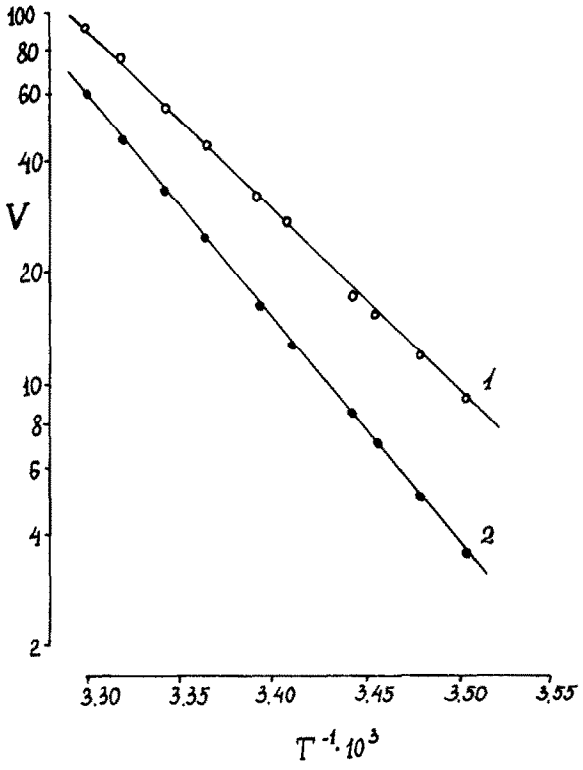


Fig.3. Arrhenius plot for Na,K-ATPase in normal conditions (1) and after 20 min preincubation of the enzyme with 0.75 mM AFSPP [2]. The reaction rate was calculated from the increase in P_i under optimal conditions; $MgCl_2$, 5 mM; ATP, 1 mM.

Since the carboxylic group in the active center of Na,K-ATPase is an immediate participant in ATP hydrolysis, a question arises whether inhibition of the activity by AFSPP results in taking the enzyme–inhibitor complex out of the reaction as can be proposed from fig.1. Fig.3 shows the Arrhenius plots for Na,K-ATPase in the control samples (curve 1) and after preincubation with 0.75 mM AFSPP, when the enzyme activity at 37°C reaches ~50% of the control value (curve 2). The conditions for ATPase activity determination allowed us to measure the maximal rate over the whole temperature range [13] and the Arrhenius plots were linear in both cases. The values of the activation energy were 17.5 ± 0.2 kcal/mol in the control and 22.9 ± 0.1 kcal/mol in case of AFSPP, i.e., they differed significantly. Thus, treatment of the Na,K-ATPase preparations by AFSPP not only inactivates but also partially modifies some of the enzyme molecules.

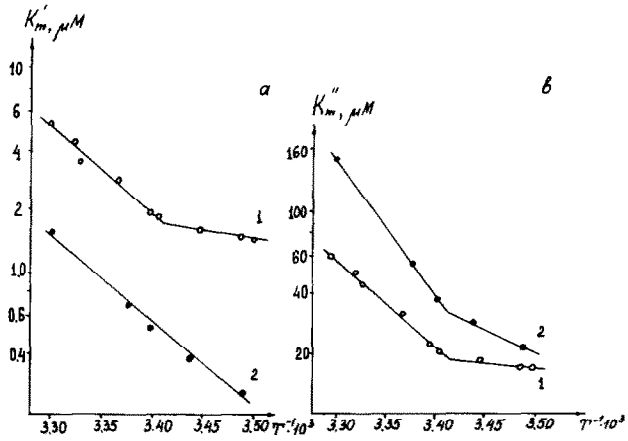


Fig.4. Arrhenius plot for two K_m values for ATP determined at low (<40 μM, a) and high (>60 μM, b) concentrations of the substrate in normal conditions (1) and after enzyme preincubation with AFSPP [2]. Conditions as in fig.1.

After preincubation of Na,K-ATPase with AFSPP the Michaelis constants for ATP are also changed. Fig.4 demonstrates the temperature dependence of both K_m values of the control and after enzyme modification by AFSPP. It is evident that AFSPP treatment causes a decrease of K'_m (at low concentrations of ATP) and an increase of K''_m (at high concentrations of ATP).

At the same time the whole pattern of the Arrhenius plots for both K_m values is changed. The increase in the activation energy of ATP hydrolysis in the presence of AFSPP (fig.3) suggests that AFSPP modifies the enzyme activity. This modification consists in an alteration of the temperature dependence of the E · I complex.

The straightening of the Arrhenius plot for K'_m values (fig.4) may indicate that the sensitivity of the complex to the phase state of the lipids is decreased.

Thus, the factors affecting the break in the Arrhenius plot for K'_m at 20°C cease to be effective in the presence of AFSPP. The second Michaelis constant, K''_m, may not have any precise physical meaning; it only reflects the allosteric action of the substrate on the oligomeric complex of the Na-pump [1–4]. Based on the assumption that the break in the Arrhenius plot for Na,K-ATPase may be interpreted in terms of the effect of lipids on the interactions in the oligomeric complex of the Na-pump [4,13], we analyzed the cooperativity coefficient (n_H) for ATP at various temperatures.

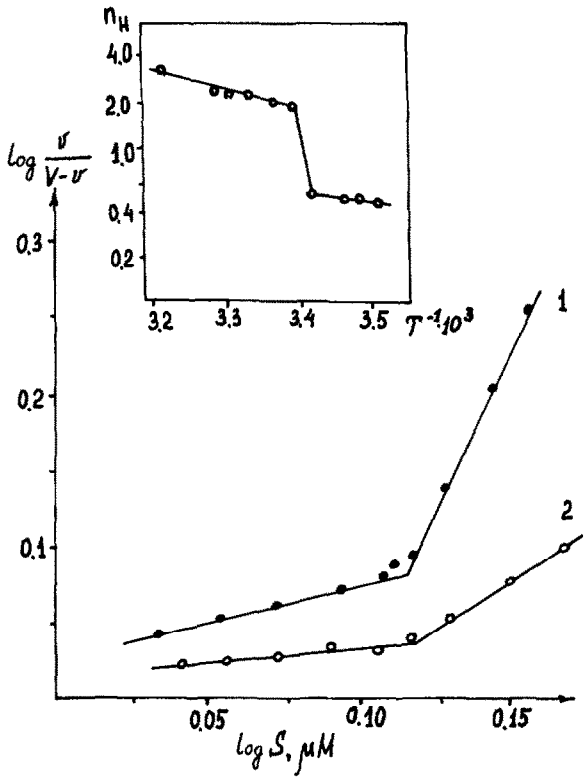


Fig.5. Determination of the Hill coefficient (n_H) for ATP at low (<40 μM) and high (>60 μM) substrate concentrations in normal conditions (1) and after 20 min enzyme preincubation with 0.75 mM AFSPP [2]. Inset: Arrhenius plot for n_H measured in the presence of high ATP concentrations.

The dependence of the Na,K-ATPase activity on ATP concentration in Hill coordinates is shown in fig.5a. ATP at 40–50 μM is critical, since n_H at lower [ATP] is <1, and at >60 μM ATP is >1. This suggests that in the case when only the catalytic sites of the enzyme having high affinity for the substrate are saturated with ATP, negative cooperative interactions may occur. An increase in ATP concentration changes the type of subunit (or protomer) interactions and results in the appearance of positive cooperativity.

In order to measure the n_H value over a wide temperature range (fig.5b) we only used ATP at 60–500 μM. From 37°C the n_H value for ATP decreased to 2 (at 22°C) and then sharply falls to 0.53. A further lowering of temperature does not significantly affect n_H. Thus, the decrease of temperature and/or ATP concentration changes the positive cooperative interactions in the oligomeric complex of Na,K-ATPase into negative ones. The temperature region when the lipid phase of the membrane becomes ordered (~20°C) is a critical one and is when such a change occurs.

AFSPP affects the sensitivity of the system to the state of the lipid environment (fig.4a); it would seem reasonable to assume that its effect is coupled with the disturbances in the cooperative interactions. Indeed, an increase from 14–30°C increases the n_H from 0.48–2.3 in the control and from 0.1–1.1 after enzyme preincubation with AFSPP (table 1). Consequently, modification of Na,K-ATPase by AFSPP prevents the appearance of positive cooperative interactions between the protomers of the Na-pump.

The Arrhenius plots for Na,K-ATPase from different preparations shows a break [1,13,15], which may be because the activity changes occur under non-optimal conditions [2]. Calculation of the true maximal reaction rate (V) gives a linear Arrhenius plot (fig.3, [2,16]). However, even under these conditions (as can be seen from fig.4), the temperature dependence of the kinetic parameters (K_m) in the Arrhenius plot is non-linear, which is due to a phase-structural rearrangement of the membrane lipids [2,13]. The changes of the cooperative properties of the system within the same temperature region (fig.5) suggest that the lipids control the interactions between the Na-pump protomers. Therefore, when no cooperativity for ATP is observed [15], this fact may be due to specificity of the lipid composition of the membrane preparation used.

Table 1
Hill coefficient (n_H) values for ATP over 60–500 μM substrate measured at different temperatures (a typical experiment)

	14°C	18°C	20°C	21°C	22°C	23°C	30°C
Control	0.48		0.53		2.05		2.30
+ AFSPP	0.10	0.60		0.90		1.00	1.10

The measurements were carried out under normal conditions or after 20 min preincubation of protein with 0.75 mM AFSPP

Based on the evidence on the oligomeric structure of the Na-pump in the membrane [1–3], we may suggest that under optimal conditions (37°C and high ATP concentration) the minimal number of protomers having a positive cooperativity, is 4. The same was concluded in [17], whereas no cooperativity for ATP was demonstrated in [15]. The decrease of ATP concentration or temperature leads to a transition of positive cooperative interactions into negative ones (probably, without change of number of protomers interacting). Presumably AFSPP interacts with the low affinity ATP-binding sites of one or two protomers of Na-pump in a similar manner to ATP, thus disturbing the allosteric effect of the substrate.

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