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Na,K-ATPase labelled with 5-iodoacetamidofluorescein: E_2-E_1 conformational transition induced by different nucleotides

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A conformational transition between E_2 and E_1 forms of Na, K-ATPase induced by different nucleotides has been studied under steady state conditions using the enzyme labelled with 5-iodoacetamidofluorescein. In the presence of K^+ the plot of fluorescence as a function of [ATP], [ADP] or [CTP] (in a range of 5 μM–12 mM) is a biphasic one. A similar dependence for AMP, ITP, GTP and UTP demonstrates a hyperbolic behaviour. The data suggest that the shift in the equilibrium between E_2 and E_1 forms of Na,K-ATPase towards the E_1 conformation is induced by ATP binding both with high and low affinity sites. Two structural features of ATP are apparently important for its interaction with more than one type of ATP binding sites or for providing for E_2–E_1 transition induced by this interaction: (i) β-phosphate group in the terminal part of the molecule, (ii) unprotonated N_1 and/or NH_2-group in the 6th position of the purine base.

Na,K-ATPase; 5-Iodoacetamidofluorescein; Conformational transition; ATP binding site

1. INTRODUCTION

During the catalytic cycle Na,K-ATPase passes through a number of distinct conformations. Spectroscopic studies as well as proteolytic experiments indicate that the enzyme can exist in two principal conformations, E_1 and E_2 [1,2]. The E_1 form predominates in the presence of Na^+ and E_2 is the major conformation with K^+.

The Na,K-ATPase mechanism is described by the scheme proposed independently by Siegel and Albers [3] and Post et al. [4]. Later on it was shown that the rate-limiting step of this cycle is E_2–E_1 conformational transition [1]. This conversion is accelerated by ATP, which interacts with a low affinity site (K_d = 0.45 mM).

It is known that besides ATP Na,K-ATPase can use other nucleotides as substrates, but only ATP or CTP hydrolysis shows a non-hyperbolic nature of the substrate-velocity curve [5]. It is proposed that the deviation from the hyperbolic kinetics is due to ATP binding with the low-affinity site on E_2-conformation which has to increase the rate of E_2-E_1 transition [6].

In this study we tried to compare the effects of different nucleotides on E_2-E_1 transition using Na,K-ATPase labelled with IAF.

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Abbreviations: IAF, 5-iodoacetamidofluorescein; IAF-enzyme, Na,K-ATPase labelled with IAF

2. MATERIALS AND METHODS

Na,K-ATPase was purified from duck salt glands [7] with spec. act. 25–30 μmol of P_i/mg of protein per min. The enzyme was labelled with IAF as described by Kapakos and Steinberg [8].

A Hitachi F-3000 fluorescence spectrophotometer was used for fluorescence experiments. Excitation and emission were performed at 494 and 516 nm, respectively. All experiments were made at room temperature in the medium containing 1 mM EDTA, 50 mM imidazole (pH 7.5) in the final volume 2.5 ml. Protein concentration was 10–20 μg/ml. Additions of KCl, NaCl and nucleotides were made from concentrated solutions to minimize dilutions.

The following procedure was used for preparation of ouabain-enzyme complex: the enzyme (2 mg/ml) was incubated 20 min at room temperature in solution containing 5 mM $MgCl_2$, 5 mM P_i, 50 mM imidazole (pH 7.5) and 1 mM ouabain. As a result of this procedure the enzyme activity was completely inhibited.

All nucleotides purchased from Reanal were recrystallized by the method of Berger [9]. Their purity was controlled by thin-layer chromatography. It was 98% for ATP and 90–95% for other nucleotides. All nucleotides were converted to the imidazole salts by passage over Dowex 50 in the H^+-form with subsequent neutralization with imidazole.

Imidazole was obtained from Pierce; ouabain, EDTA and sucrose were from Sigma. IAF was purchased from Molecular Probes. The other reagents were at least reagent grade from Reachim, USSR.

3. RESULTS AND DISCUSSION

It is known that IAF covalently labels Na,K-ATPase without inhibiting its enzymatic activity. The IAF-enzyme demonstrates the specific fluorescence changes associated with E_2–E_1 conformational transition [8].

The fluorescence response of IAF-enzyme on the K^+ and Na^+ is illustrated in Fig. 1. An addition of K^+ (5–80 μM) decreases the fluorescence, its minimal level being observed in the presence of 60–80 μM of K^+

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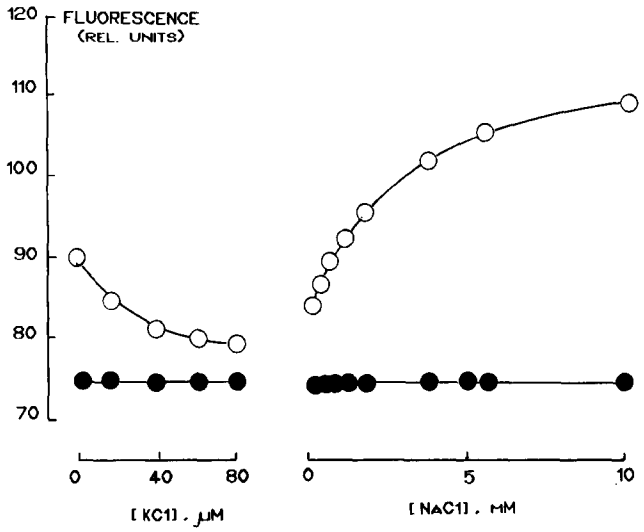

Fig. 1. The change of fluorescence of IAF-labelled Na,K-ATPase after addition of different concentrations of NaCl and KCl. (○) Native IAF-enzyme; (●) IAF-enzyme after treatment by ouabain in the presence of P_i and $MgCl_2$.

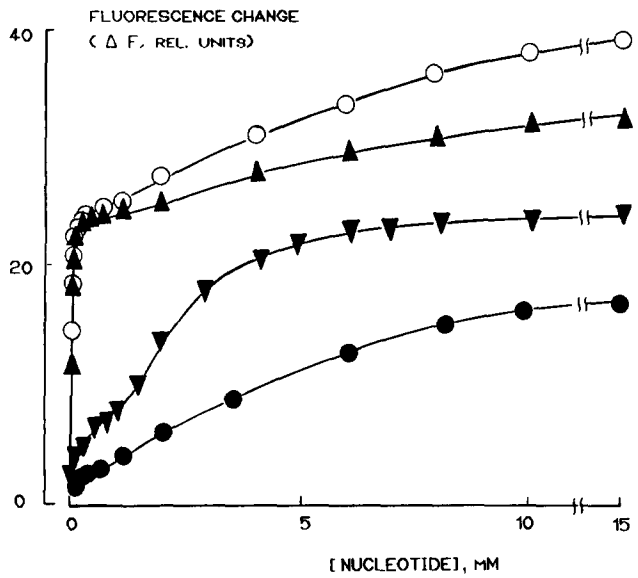

Fig. 2. The dependence of fluorescence change of IAF-enzyme upon the nucleotide concentration: (○) ATP with native enzyme; (●) ATP with the enzyme after treatment with ouabain; (▲) ADP; (▼) CTP (both with native enzyme).

(E_2-conformation). When increasing concentrations of Na^+ (up to 50 mM) were added to the IAF-enzyme the fluorescence was increased to a level higher than the original one (E_1-conformation).

Ouabain treatment of IAF-enzyme in the presence of Mg^{2+} and P_i completely abolished the fluorescence response to K^+ as well as to Na^+ (Fig. 1). The difference between fluorescence of E_1 and E_2 forms of IAF-enzyme was approximately the same for all enzyme preparations studied (35–40 relative units, $n = 6$).

When increasing concentrations of ATP were added to the E_2 form the fluorescence of IAF-enzyme was increased, reaching an intermediary plateau between 0.25 and 0.7 mM, and then increased more slowly (Fig. 2), indicating the presence on the enzyme of at least two types of ATP binding sites. The upper level of fluorescence in the presence of saturating ATP concentrations was approximately equal to that in the presence of 50 mM of Na^+.

The occurrence of two types of ATP binding sites on E_2-conformation was shown earlier [10]. It was assumed, however, that the facilitation of E_2–E_1 transition was a result of ATP binding only with a low-affinity site [1]. In accordance with our previous results [11], we found that considerable shift in the equilibrium between E_2 and E_1 conformations is due to high affinity binding of ATP. The same evidence was obtained by Kapakos and Steinberg [8]. In the present investigation we also found that saturation of low affinity site (or sites) by ATP leads to the additional increase in E_1 conformation.

Treatment of IAF-enzyme with ouabain almost completely blocked fluorescence change induced by ATP at concentrations lower than 0.3 mM, but did not abolish the fluorescence response to higher ATP concentrations. This is in agreement with the observations of Askary et al. [12], who found that the enzyme-ouabain complex also had two types of ATP binding sites. Apparently ouabain binding to Na,K-ATPase does not block ATP binding with both sites, but abolishes conformational changes induced by high affinity ATP binding.

ADP, AMP, CTP, ITP, GTP and UTP also provide E_2–E_1 transition, but the maximal fluorescence change observed in the presence of saturating concentrations of these nucleotides is less than that in the presence of saturating concentrations of ATP (respectively 80, 70, 55, 30, 20 and 16%). At the same time the biphasic

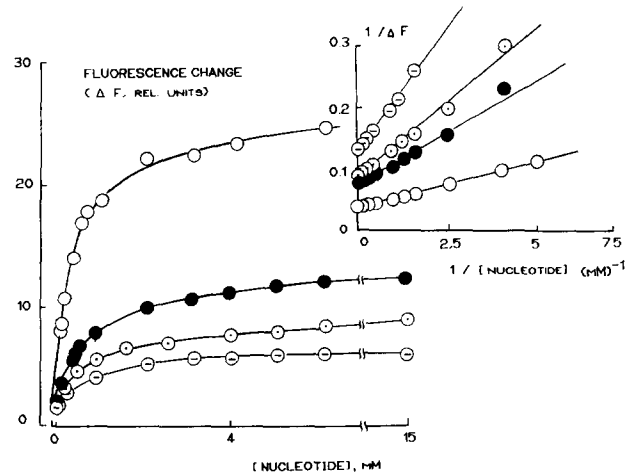

Fig. 3. The dependence of fluorescence change of IAF-enzyme upon the nucleotide concentration (usual plot and double reciprocal plot): (○) AMP; (●) ITP; (⊙) GTP; (○) UTP.

curve for fluorescence change vs nucleotide concentrations was observed only with 3 nucleotides: ATP, ADP and CTP (Fig. 2). The similar plots for AMP, ITP, GTP and UTP were hyperbolic (Fig. 3) with K_a values equal to 0.38, 0.44, 0.55 and 0.67 mM, respectively. We can conclude that nucleotides which are hydrolysed in accordance with nonhyperbolic kinetics (ATP and CTP) demonstrate also a complex behaviour in fluorescence experiments. These data support the suggestion that nonhyperbolic kinetics of nucleotides hydrolysed by Na,K-ATPase is a result of their action on E_2–E_1 transition.

When ATP and ADP concentrations are less than 0.3 mM both nucleotides induced equal fluorescence response (Fig. 2), but at higher concentrations ATP is a little bit more effective. A relatively small increase in fluorescence of IAF-enzyme was observed at low CTP concentrations (the first phase of the curve), but the increment of fluorescence in the second phase was even higher than that for ATP.

A comparison of the structure of ATP and ADP with their effects on E_2–E_1 transition indicates that the γ-phosphate group of nucleotide is not important neither for its high affinity binding nor for providing for conformational change induced by this binding. On the other hand this group is important for such an effect of the nucleotide, which is due to its binding with low affinity site. The removal of the β-phosphate group transforms the nucleotide effect: the E_2–E_1 transition induced by AMP is the result of nucleotide binding only with one type of sites.

It is evident from our results that not only the terminal part of nucleotide molecule, but also the structure of its nitrogenous base plays an important role in binding or in providing for E_2–E_1 conversion. Only nucleotides containing adenine or cytidine can provide this transformation through interaction with both nucleotide binding sites.

As we discussed recently [13] adenine and cytidine at physiological pH had two common properties: (i) both bases exist in amino form having NH_2-group in sixth (fourth) position, (ii) both bases possess non-protonated nitrogen (N_1 or N_3) in their nitrogenous ring. It is likely that the presence of such a structure in nucleotide molecule may provide either appropriate affinity to binding sites or take part in the induction of conformational transition, which is due to nucleotide binding with these sites.

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