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The role of carboxyl groups of Na^+/K^+-ATPase in the interaction with divalent cations

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Mg^{2+}-induced subconformational changes of the E_1 conformation of partly purified pig kidney Na^+/K^+-ATPase were studied by fluorescence techniques. In the enzyme with carboxyl groups modified by carbodiimide in the presence of an exogenous nucleophile the efficiency to pass through conformational substates was substantially lower than in the unmodified enzyme. Magnesium could form bridges between carboxyl groups near the membrane/water interface and negatively charged phospholipid polar heads.

Na^+/K^+-ATPase is a membrane-bound enzyme responsible for the active transport of potassium and sodium across the plasma membrane. It consists of two subunits, the catalytic α-subunit and the β-subunit, which is a glycoprotein of unknown function. During ion transport, the enzyme is believed to proceed through two major conformational states E_1 and E_2 characterized by different affinity for potassium and sodium [1]. Within the framework of the two conformational states, the enzyme passes through different substates [2], in the formation of which divalent cations can play an important role either by binding to the protein binding sites [3] or, indirectly, through interaction with phospholipids [4,5].

Recently, an inhibition of Na^+/K^+-ATPase and K^+-dependent *p*-nitrophenylphosphatase by carbodiimide was shown, together with a protective effect of added nucleophiles [6]. The carboxyl groups remained modified in the latter case but no intramolecular cross-linking that would cause enzyme inhibition, occurred.

In the present work we modified FITC-labelled Na^+/K^+-ATPase (fully saturated by 3 mM magnesium) with EDC in the presence of ethanolamine and studied the effect of magnesium on the conformational substates of the enzyme by measuring steady-state fluorescence anisotropy changes.

The enzyme from pig kidney outer medulla was purified by the simple method of Jørgensen [7]. The activity of the enzyme was 0.9–1.1 mmol of phosphate per mg protein per hour, determined according to [8]. The K^+-dependent *p*-nitrophenylphosphatase activity was measured at 37°C in total volume of 1 ml, containing 50 mM imidazole, (pH 7.2), 20 mM KCl, 5 mM MgCl, 1 mM EGTA and 50–80 μg protein. The reaction was started by adding 3 mM *p*-nitrophenyl phos-

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; FITC, fluorescein 5-isothiocyanate; DPH, 1,6-diphenyl-1,3,5-hexatriene; pNPPase, *p*-nitrophenylphosphatase.

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phate as described in Ref. 9. FITC-labelled Na^+/K^+-ATPase was prepared according to Ref. 10.

The carboxyl groups of the Na^+/K^+-ATPase were modified with EDC and ethanolamine as described by Yamada et al. [11].

Steady-state anisotropy measurements were done in 0.25 M sucrose, 25 mM imidazole and 5 mM KCl (pH 7.2), using a MPF 3L spectrofluorometer (Perkin-Elmer). The anisotropy values were corrected for the scattering of the excitation beam, using a glycogen blank of the same absorbance. In addition, to correct for the depolarization due to several reflections both before absorption and before reaching the detector, the true anisotropy values r were calculated from observed ones (r') and the absorbances A according to Cavatorta et al. [12]:

$$r = \frac{r'}{1 - K \cdot A}$$

where K was the actual proportionality constant. The absorbance was maintained below 0.1 (λ = 530 nm) for all samples. The excitation wavelengths for tryptophan and FITC were, respectively, 295 nm and 494 nm, the emission wavelengths were 340 and 519 nm. The Förster energy transfer efficiency between the tryptophan residue as donor and FITC as acceptor could be determined from the decrease of the emission intensity of tryptophan according to $\varepsilon = E_1/E_2$ where E_1 is the intensity in the presence and E_2 in the absence of acceptors.

The fluorescence probe DPH was dissolved in acetone and membranes were labelled according to Ref. 13. The excitation and emission wavelengths were 360 nm and 450 nm, respectively. The fluorescence lifetime was measured by the phase-modulation method T-format, this spectrofluorometer was used for measurements of both the differential tangent and the steady-state anisotropy. Weber's theory of hindered rotation [14] was used, yielding the limiting anisotropy r_∞ and the rotational relaxation time τ_c.

EDC was obtained from Fluka, all other chemicals were purchased from Sigma.

The effect of magnesium on the conformational substates of Na^+/K^+-ATPase was measured under conditions favouring the E_2 state. Using the intrinsic fluorescence of tryptophan residues, we followed the changes of anisotropy from 0.107 ± 0.003 in the absence of magnesium to 0.119 ± 0.002 in the presence of 3 mM $MgCl_2$; indicating a switch between the substates of the E_1 conformation. Because the total fluorescence intensity was not changed after the addition of magnesium (data not shown), the enzyme apparently remained in the E_1 state only. Furthermore, we labelled Na^+/K^+-ATPase with FITC, a preferential label of lysine groups at pH 9.0, and observed an increase, (approx. 5%) in the Förster energy transfer between tryptophan residues as donors and FITC molecules as acceptors in the presence of 3 mM $MgCl_2$ (data not shown). The increase of efficiency of Förster energy transfer indicated a decrease of the average distance between acceptors and donors in the presence of divalent cations.

Besides these energy subconformational changes we followed the effect of divalent cations on the phospholipid phase. An Na^+/K^+-ATPase-enriched membrane fraction was incubated with the hydrophobic fluorescent probe DPH and limiting anisotropy (r_∞) values were estimated using the phase cross-correlation subnanosecond spectroflu-

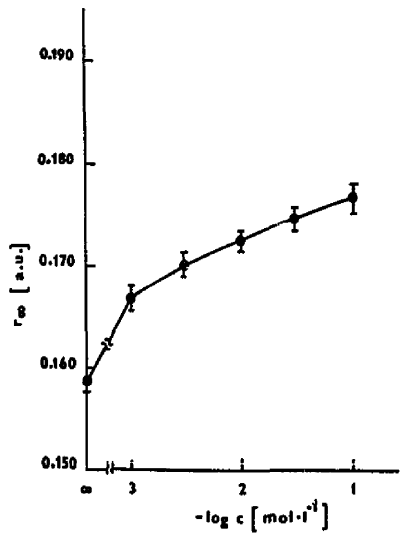

Fig. 1. The effect of magnesium on limiting anisotropy (r_∞) of DPH incorporated into Na^+/K^+-ATPase-enriched membrane fraction. The values with standard errors are averages from six measurements of three independent experiments.

orometer SLM 4800 S and Weber's theory of hindered rotation [15]. Fig. 1 shows an increase of limiting anisotropy of DPH embedded in phospholipids after magnesium treatment in the concentration range where the enzyme subconformational changes were also observed.

Finally, carboxyl residues on Na^+/K^+-ATPase were transformed by carbodiimides in the presence of exogenous nucleophile. The remaining activity of Na^+/K^+-ATPase and pNPPase after reaction of EDC plus ethanolamine was about 80% (Na^+/K^+-ATPase) and 85% (pNPPase) of the initial enzyme activity. As shown in Fig. 2 the fluorescence anisotropy of FITC-labelled Na^+/K^+-ATPase gradually increased with magnesium concentration, suggesting again different substates of the E_1 conformation. In addition, the anisotropy of FITC-labelled, but further unmodified enzyme, increased faster with magnesium ion concentration than did the anisotropy of protein treated with EDC and ethanolamine.

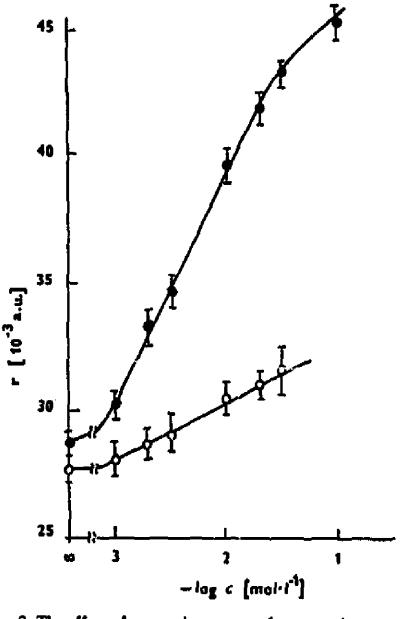

Fig. 2. The effect of magnesium on steady-state anisotropy (r) of FITC-labelled Na^+/K^+-ATPase. Untreated (●) and treated with 1 mM EDC and 100 mM ethanolamine (○). The values with standard errors are averages from six measurements of three independent enzyme modifications with EDC plus ethanolamine.

TABLE I

THE EFFECT OF MAGNESIUM ON STEADY-STATE ANISOTROPY OF TRYPTOPHAN RESIDUES OF Na^+/K^+-ATPase (r_{Trp}) IN ARBITRARY UNITS (A.U.) AND EFFICIENCY OF FÖRSTER ENERGY TRANSFER (ε) BETWEEN TRYPTOPHAN RESIDUES AND ENZYME-BOUND FITC MOLECULES

The values with standard errors are averages from six measurements of three independent experiments.

Concentration of Mg^{2+} (mM)	r_{Trp} (a.u.)	ε (%)
0	0.107±0.003	7.5
3	0.119±0.002	14.0

Consequently, what role in setting up the conformational substates do carboxyl groups play? First of all, the modification of carboxyl groups can effectively suppress Mg^{2+}-induced enzyme subconformational changes. Carboxyl groups are, therefore, evidently involved in cation-binding sites on the enzyme. Because in four of eight α-subunit transmembrane spans dicarboxylic amino acids (glutamate and aspartate) create negatively charged residues on the outer side of the membrane/water interface [17], we proposed a novel role for the Mg^{2+} action. Namely, this ion could form a bridge between negative carboxyl groups of the enzyme and negatively charged phospholipid polar heads.

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