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Mg^{2+}-induced changes of lipid order and conformation of $(Na^+ + K^+)$-ATPase

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The effect of magnesium on the phospholipid order parameter and not the conformation of purified pig kidney outer medulla $(Na^+ + K^+)$-ATPase was investigated by fluorescence techniques. Measurements with a fluorescent probe TMA-DPH and its sensitized fluorescence with tryptophan residues as donors revealed that magnesium increased the order of the membrane phospholipids both in the lipid annulus and in the bulk phase. Changes in the lipid order induced by Mg^{2+} can be closely referred to the protein arrangement followed by the steady-state anisotropy of FITC-labeled $(Na^+ + K^+)$-ATPase.

Introduction

The active transport of sodium and potassium across the plasma membrane of animal cells is catalyzed by $(Na^+ + K^+)$-ATPase, an enzyme specifically inhibited by cardiac glycosides [1]. During the transport cycle the enzyme passes through several conformational substrates induced by binding of different ligands (e.g., ATP, Na^+, K^+, Mg^{2+}). Besides this, $(Na^+ + K^+)$-ATPase activity is affected by polyunsaturated fatty acids [2], vanadium [3] and some other substances that have been also reported to influence phospholipid parameters described in terms of fluidity or order parameter [4]. Simultaneously, $(Na^+ + K^+)$-ATPase activity is sensitive to the composition and structural organization of membrane lipids in its closest neighborhood [5]. It seems, therefore, likely that, besides the direct interaction of several ligands (Na^+, K^+, ATP, ouabain, P_i) with catalytic sites located on the α-subunit, the enzyme activity could be modulated by lipid–protein interactions.

In the present paper, an analysis of the action Mg^{2+} on $(Na^+ + K^+)$-ATPase is reported. Mg^{2+} is known to be required for full activity of $(Na^+ + K^+)$-ATPase [6], but modulations of enzyme activity were observed at concentrations of Mg^{2+} high enough to detect also the decrease of membrane fluidity [4].

The question remained, to what extent could the modulating effects of Mg^{2+} be interpreted by direct magnesium binding to the protein and to what extent are they due to the indirect action of lipid–protein interactions. Therefore, we analysed the effect of magnesium on phospholipid parameters using nanosecond fluorescence spectroscopy of TMA-DPH. These results were related to the conformational changes of $(Na^+ + K^+)$-ATPase

Abbreviations: FITC, fluorescein isothiocyanate; TMA-DPH, 1-(4-trimethylammonium)phenyl-6-phenyl-1,3,5-hexatriene; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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monitored by changes of the steady-state anisotropy of the enzyme labeled with FITC and to the quenching of tryptophans naturally occurring in the protein by a charged iodide quencher.

Materials and Methods

$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) from pig kidney outer medulla was prepared by Jørgensen's procedure [7]. The specific activity of the enzyme was 0.9–1.1 mmol phosphate per mg protein per h determined according to Ref. 9. FITC-labeled $(Na^+ + K^+)$-ATPase was prepared according to Ref. 9 as follows. 1 mg protein was incubated at pH 9.2 in 100 mM Tris-HCl/1 mM EDTA at room temperature in the dark for 6 h with 5 μM FITC (stock solution in dimethyl sulfoxide). The labeled enzyme was then diluted 10-fold with 25 mM imidazole (pH 7.0)/1 mM EDTA and dialyzed overnight.

Freeze-thaw sonication of the cholate-solubilized enzyme was done to reconstitute of the FITC-labeled enzyme into the phospholipid liposomes according to Ref. 10. The total phospholipids were determined as in Ref. 11. Proteins were determined by the method of Lowry et al. [12].

Labeling of samples with TMA-DPH was done according to Kuhry et al. [13]. All measurements were performed at 22.0 ± 0.3° C. The excitation wavelength was 360 nm and fluorescence emission was isolated from Rayleigh and Raman scattering by using a Scott KV 420 filter.

For the estimation of structural and dynamic phospholipid parameters, Weber's theory of hindered rotations [14] was used, yielding the limiting anisotropy, r_∞, and the rotational relaxation time, τ_c. Eqn. 1 applies for these quantities:

$$r = (r_0 - r_\infty)/(1 + \tau/\tau_c) + r_\infty \quad (1)$$

where r is the steady-state anisotropy, r_0 the fluorescence anisotropy observed in the absence of depolarizing factors ($r_0 = 0.395$) and τ is the fluorescence lifetime. The order parameter, S_∞, depends on the limiting anisotropy, r_∞, as $S_\infty = (r_\infty/r_0)^{1/2}$. To yield the above values, the steady-state anisotropy, differential tangent, $\mathrm{tg}\Delta$, and fluorescence lifetime were measured by the cross-correlation phase and modulation spectrofluorometer SLM 4800 S [15] operating in the T-format and were processed according to Eqn. 2 [16]:

$$(2R\tau)^2 \cdot (m \cdot \mathrm{tg}\Delta) + (2R\tau) \cdot (C \cdot \mathrm{tg}\Delta - A) + (D \cdot \mathrm{tg}\Delta - B) = 0 \quad (2)$$

where

$$A = 3B = \omega\tau(r_0 - r),\ C = 1/3 \cdot (2r - 4r^2 + 2),$$

$$D = 1/9 \cdot (m + m_0\omega^2\tau^2),\ m = (1+2r)(1-r)$$

and where R is the rotational relaxation rate and ω the modulation frequency. All steady-state measurements were carried out with the radio-frequency electronics switched off.

The excitation wavelength for tryptophan residues was 280 nm and the emission wavelength was 330 nm. For the analysis of the tryptophan quenching process in $(Na^+ + K^+)$-ATPase we supposed independent, equally absorbing, fluorophores. Although quantum yields can vary, adherence to the Stern-Volmer law [17] for each fluorophore was assumed. Besides this, we considered all the tryptophan residues buried in the membrane to be totally inaccessible to KI and the others to be accessible with the same value of the Stern–Volmer constant, K_Q. In such a condition, the classical Stern–Volmer equation becomes [18]:

$$F_0/(F_0 - F) = 1/f_a \cdot K_Q \cdot [Q] + 1/f_a \quad (3)$$

where F_0 and F are fluorescence intensities without and with the quencher (KI), respectively, [Q] is the concentration of the quencher and f_a is the fractional maximum accessible protein fluorescence. From Eqn. 3, a plot of $F_0/(F_0 - F)$ vs. $1/[Q]$ yields a straight line of slope $1/(F_a \cdot K_Q)$ and intercept $1/f_a$, with K_Q being equal to intercept/slope).

Measurements of energy transfer from tryptophan residues to TMA-DPH were carried out on the SLM 4800 S spectrofluorometer (with radio frequency switched off) in a fluorescence cuvette containing 2 ml buffer solution. The total phospholipid content was kept constant and equal to 100 μg/ml. TMA-DPH in acetone was added with a 2 μl glass micropipet under stirring. Spectroscopic measurements were then performed after a

5 min equilibration ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 330$ nm). After each experiment, the emission from vesicles labeled with the same amount of TMA-DPH, but without the protein, was recorded for correction.

The energy-transfer efficiency (E) was calculated from the reduction of donor emission according to Eqn. 4:

$$E = 1 - Q_T/Q_0 = R_0^6/(R_0^6 + r^6) \quad (4)$$

where Q_T and Q_0 are the quantum yields of donor (tryptophan) in the presence and in the absence of acceptor (TMA-DPH), respectively, r is the average distance between fluorophores and R_0 is the distance at which the rate of excitation energy transfer between the donor and acceptor is equal to the sum of the rates of all other modes of deactivation, and is given by Eqn. 5:

$$R_0 \doteq 9.8 \cdot 10^{-6} \cdot (J \cdot k^2 \cdot Q_0 \cdot n^4)^{1/6} \quad (5)$$

where J is the spectral overlap integral, k^2 the dipole-dipole orientation factor (its value is 2/3 when complete rotational depolarization takes place during the molecular lifetime) and n the refractive index of the medium.

The anisotropy of sensibilized fluorescence of TMA-DPH (with tryptophan residues as donors) was measured by one channel on SLM 4800 S ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 430$ nm). The anisotropy value was calculated according to Eqn. 6:

$$r = (K \cdot I_{\parallel}/I_{\perp} - 1)/(K \cdot I_{\parallel}/I_{\perp} + 2) \quad (6)$$

where $I_{\parallel}$ and $I_{\perp}$ are intensities polarized parallel and perpendicular to the polarization of the excitation beam and K is an instrument correction factor. Before each experiment the intensities without TMA-DPH were recorded for correction.

Results

The ordering of bulk phospholipids by Mg^{2+}

Freshly prepared phospholipid liposomes and partly purified ($Na^+ + K^+$)-ATPase from pig kidney outer medulla were used for measurement of the influence of Mg^{2+} in physiologically relevant concentrations (10^{-5} to 10^{-2} M) on membrane phospholipids. Fig. 1 shows the effect of Mg^{2+} on the steady-state anisotropy of TMA-DPH. Mg^{2+}

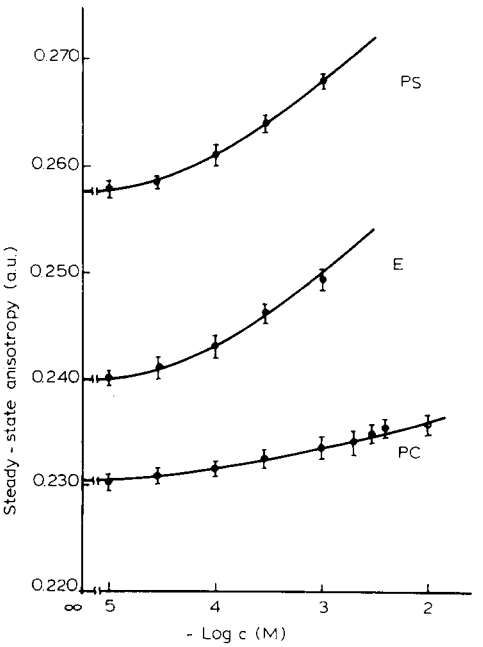

Fig. 1. Steady-state anisotropy of TMA-DPH incorporated into phosphatidylserine liposomes (PS), phosphatidylcholine liposomes (PC) and ($Na^+ + K^+$)-ATPase membranes from pig kidney outer medulla (E) as a function of concentration of Mg^{2+} (log c); $t = 22.0 \pm 0.3$° C.

in the millimolar range increased the steady-state anisotropy of TMA-DPH in both phosphatidylserine and phosphatidylcholine liposomes. However, the increase in anisotropy is more pronounced in phosphatidylserine liposomes, probably due to their more negative surface charge. Different absolute values of TMA-DPH anisotropy are the consequence of different composition in terms of both fatty acids and polar heads. The anisotropy of TMA-DPH in ($Na^+ + K^+$)-ATPase increased already in submillimolar concentrations of Mg^{2+}. Nevertheless, the fluidity or microviscosity of membranes often estimated from the steady-state anisotropy of TMA-DPH is inadequate to describe the probe motion in the membrane because of non-zero limiting anisotropy. A resolution into the structural and dynamic parts characterized by limiting anisotropy and rotational relaxation time, respectively, seemed to be justified.

Using the phase method we separated these two contributions to the steady-state anisotropy of

TMA-DPH according to Eqn. 1. While the rotational relaxation time remained without any change up to the 10 mM of magnesium (data not shown) the order parameter or the lateral pressure (see Ref. 19) rapidly increased (Fig. 2).

The effect of Mg^{2+} on boundary phospholipids

The negatively charged phosphatidic acid and phosphatidylserine are assumed to have preferential affinity for $(Na^+ + K^+)$-ATPase [20]. It seems probable that divalent cations can bind in the close neighborhood of the protein (in the lipid annulus). Measurement of the sensitized fluorescence of TMA-DPH excited by tryptophans of $(Na^+ + K^+)$-ATPase to TMA-DPH incorporated into the membrane was carried out. Because the Förster energy transfer can occur over distances of several nanometers only, the sensitized fluorescence of TMA-DPH must reflect preferentially the properties of the lipid annulus. On assuming homogeneously distributed TMA-DPH and the membrane to be a lattice with a lattice constant of 0.7 nm and a tryptophan residue at a distance of 0.4 nm from the half-plane of phospholipids, then TMA-DPH distributed in the first shell contributed by almost 30% and in the second shell by approx. 20% to the total intensity of sensitized fluorescence. Fig. 3 shows that Mg^{2+} sharply increased the steady-state anisotropy of TMA-DPH sensitized by tryptophans. We also calculated the critical separation distance, $R_0 = 3.1$ nm (see Table I), and the average distance between tryptophan residues and TMA-DPH before and after Mg^{2+} treatment (see Table I). The average concentration of TMA-DPH was 0.4 μM; that of protein 100 μg/ml. However, both TMA-DPH and tryptophans in $(Na^+ + K^+)$-ATPase were

TABLE I

EFFICIENCY OF ENERGY TRANSFER, E, AND THE AVERAGE DISTANCE, r, BETWEEN TRYPTOPHAN RESIDUE AND TMA-DPH

The value $R_0 = 3.1$ nm was calculated according to Eqn. 5.

$[Mg^{2+}]$ (mM)	Efficiency of energy transfer (E) (%)	Average distance (r) (nm)
0	15.0	4.14
2	16.7	4.05

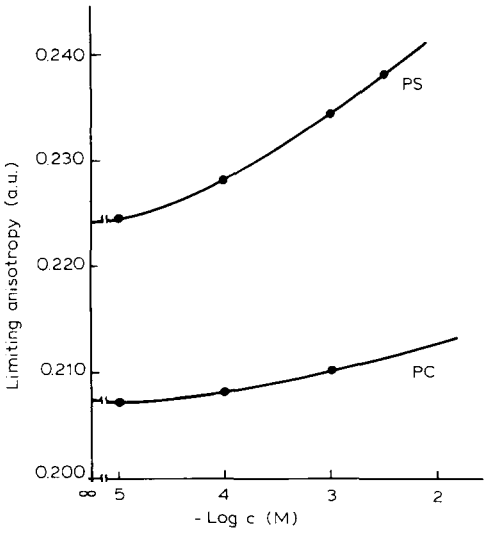

Fig. 2. Limiting anisotropy of TMA-DPH (calculated according to Ref. 10) in phosphatidylserine (PS) and phosphatidylcholine (PC) liposomes, respectively, at different concentrations of Mg^{2+}. The rotational relaxation time remained unchanged within the experimental errors range at all concentrations; $t = 22.0 \pm 0.3$° C.

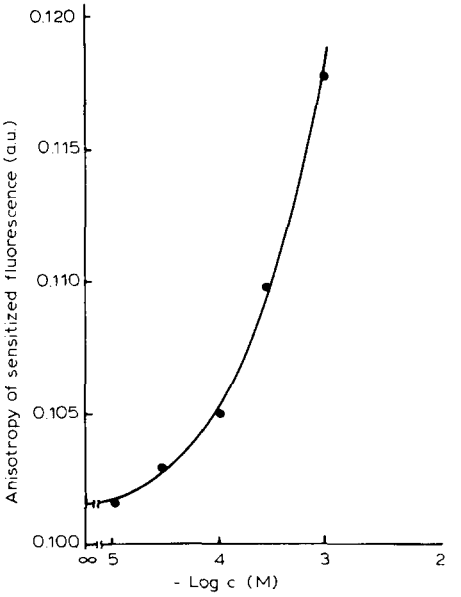

Fig. 3. Influence of Mg^{2+} on the anisotropy of sensitized fluorescence of TMA-DPH (with tryptophan residues of $(Na^+ + K^+)$-ATPase membranes as donors).

concentrated in membranes because the ratio of total cuvette and membrane volumes was approx. 1000 : 1. If we took this fact into account, the effective concentrations of both TMA-DPH and tryptophan residues (12 tryptophan residues (12 tryptophan residues per α-subunit of enzyme [21] reached several millimoles per liter. The Förster theory of the effect of acceptor concentration upon fluorescence quantum yield assumes that no energy migration occurs between donor molecules (of the same kind) so that the following condition is fulfilled:

$$y = c_D/c_A \cdot (I_{\nu(D,D)}/I_{\nu(D,A)})^{1/2} \ll 1 \quad (7)$$

where c_A and c_D are the acceptor and donor concentrations, respectively, and $I_{\nu(D,D)}$ and $I_{\nu(D,A)}$ are the donor-donor and donor-acceptor overlap integrals, respectively [22]. Because, in our case, both the overlap integrals and the donor and acceptor concentrations were comparable, condition [7] was not fulfilled. An increase in y is accompanied by an enhancement of the probability of excitation energy migration between identical molecules. Therefore, one must consider the possibility that the donor molecules (in our case tryptophan residues) mediate a radiationless excitation energy transfer to the acceptors (TMA-DPH). Furthermore, the concentration quenching is accompanied by a decrease in the mean lifetime of the molecular excited state and thus an increase in the fluorescence anisotropy of the donor. The anisotropy value of tryptophan residues in ($Na^+ + K^+$)-ATPase was $r(0.099 \pm 0.001)$. Although the donor anisotropy was not zero, this does not justify conclusions about the anisotropic arrangement of the residues or permit one to estimate the order parameter. However, even if one assumes an isotropic orientation of tryptophan residues, the anisotropy of sensitized fluorescence of TMA-DPH with tryptophans as donors would be higher than the anisotropy of TMA-DPH excited by nonpolarized light due to the higher order parameter of phospholipids in the annulus. Our measurements indicated that the anisotropy of sensitized fluorescence ($r = 0.103$) was approximately the same as the anisotropy of TMA-DPH excited by nonpolarized light ($r = 0.101$).

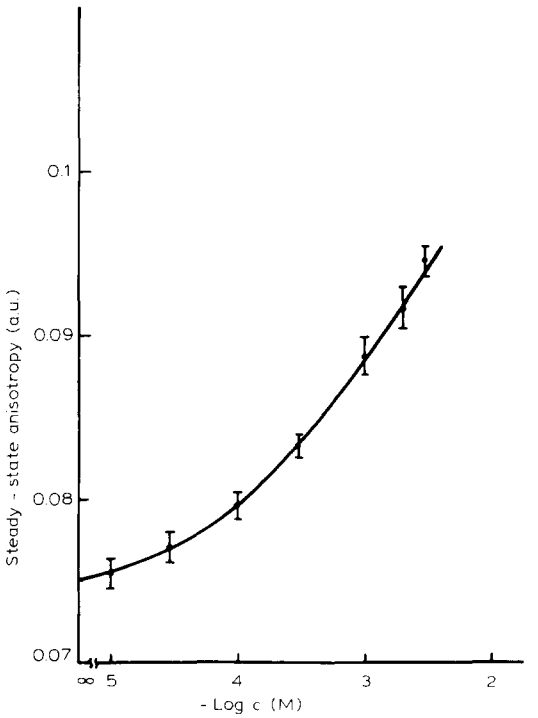

Fig. 4. Steady-state fluorescence anisotropy of FITC-labeled ($Na^+ + K^+$)-ATPase membranes from pig kidney outer medulla as a function of Mg^{2+} concentration.

Protein structural rearrangement

Mg^{2+} in millimolar concentrations increased the order parameter of TMA-DPH both in bulk phospholipids and in the lipid annulus (Figs. 1, 3). Nevertheless, the question remained whether such changes were connected with changes in protein conformation. We labeled ($Na^+ + K^+$)-ATPase with FITC and followed its fluorescence anisotropy at different concentrations of Mg^{2+}. Fig. 4 shows that also the anisotropy of FITC increased after Mg^{2+} treatment, indicating a motion restriction of the probe. To obtain more information on normal movement with respect to the membrane plane, we followed the collisional quenching of tryptophan residues with KI. Modified Stern–Volmer plots of quenching in with and without 2 mM Mg^{2+} are shown in Fig. 5. The intercepts suggest that the presence of magnesium resulted in a slightly higher effective Stern–Volmer constant and simultaneously in a greater portion of tryptophan residues fully accessible to KI.

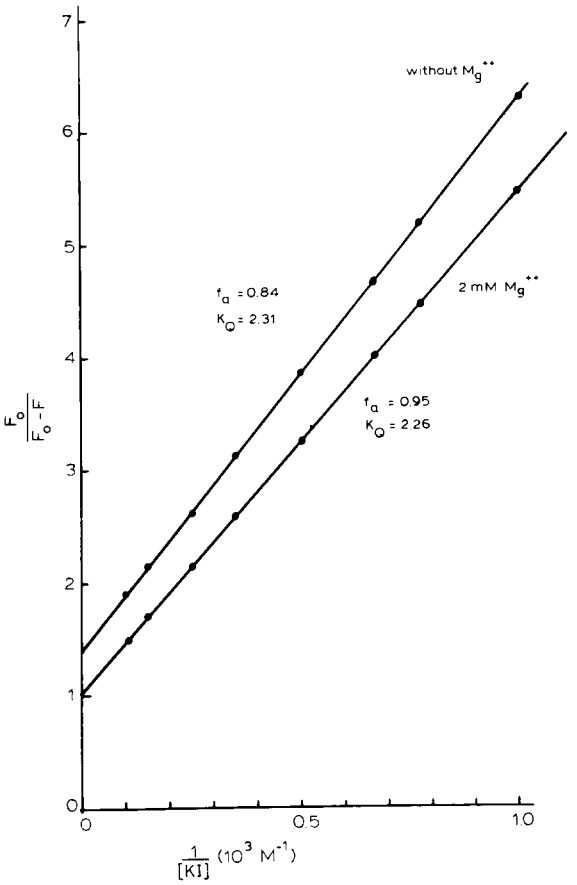

Fig. 5. A modified Stern–Volmer plot of collisional quenching of tryptophan residues from $(Na^+ + K^+)$-ATPase with KI as a quencher (a) in the absence of Mg^{2+}, yielding 84% of accessible tryptophan residues and $K_Q = 2.31$ nmol^{-1} and (b) in the presence of 2 mM Mg^{2+}, yielding 95% of accessible tryptophan residues and $K_Q = 2.26$ mmol^{-1}.

Discussion

An early study [4] suggested that cations such as Mg^{2+}, Ca^{2+}, Na^+ and K^+ interact primarily with the polar headgroups of the phospholipids in purified $(Na^+ + K^+)$-ATPase, inducing structural changes in the lipid vesicles. Addition of divalent cations to lipid is known to decrease the fluidity of the bilayer structure [23], in contrast to the cations which are known to displace divalent cations from the polar headgroup binding sites [4], resulting in an increase of fluidity. However, the steady-state anisotropy of hydrophobic probes (DPH, TMA-DPH) from which fluidity were often estimated, included contributions of both the structural properties of the bilayer (expressed as limiting anisotropy, r_∞) and its dynamic state (expressed as the rotational relaxation time, τ_c).

Our results show that Mg^{2+} increased the limiting anisotropy of phospholipids in millimolar concentrations, while the rotational relaxation time, in agreement with previous results [3], remained unchanged within the experimental error limits. The more expressed effect of Mg^{2+} on phosphatidylserine than on phosphatidylcholine is probably due to the more negative surface charge and higher Mg^{2+} binding by the first phospholipid.

The second topic of the present paper was to clarify the role of lipid annulus in the acting of Mg^{2+} on $(Na^+ + K^+)$-ATPase. Protein boundary lipids are usually believed to be highly ordered structures [24] and they might stabilize the conformation of the protein and protect the protein from the effect of changes in the bulk phase. The question was whether the ordering effect of Mg^{2+} would manifest itself in the lipid annulus. Sensitized fluorescence of TMA-DPH with tryptophan residues as donors (reflecting the property of lipids from the nearest protein environment) showed that bulk and boundary lipids did not differ significantly in their order, as was also theoretically predicted recently [25]. It is also possible, however, that the more ordered lipids exist only in a very narrow ring of one molecule thickness around the protein, as was supposed earlier [20]. In any case, Mg^{2+} significantly increased the anisotropy of sensitized fluorescence of TMA-DPH, suggesting a high ordering in the lipid annulus after Mg^{2+} treatment. Clearly, changes in the lipid structural parameters may but need not to be reflected in the structure of the protein. Measurements with FITC-labeled enzyme revealed a close relationship between the increase of the order parameter (Figs. 1, 2) and the rotational restriction of FITC, resulting in higher anisotropy values (Fig. 5) and indicating a more stable protein conformation after Mg^{2+} treatment due to the higher order of lipids in the annulus. Besides this, the bridges created by Mg^{2+} on the negatively charged heads of phospholipids led to an increase in the average lipid–lipid exchange lifetime and caused the slowing down of phospholipid diffusion in the

lipid annulus. Both these effects favor the stabilization of protein conformation, probably necessary for a fully active complex.

The collisional quenching of protein tryptophan residues by KI showed that after 2 mM Mg^{2+} treatment 94% of the tryptophan residues are fully accessible to KI, compared with 83% of residues in the absence of Mg^{2+}, which supports the view of the normal movement (with respect to the membrane plane) of the protein during its conformational change caused by Mg^{2+} [26]. From the primary structure of kidney $(Na^+ + K^+)$-ATPase α-subunit [21,27] it follows that 10 tryptophan residues occur in the hydrophobic parts of the molecule, while only Trp-100 and Trp-926 are buried in the membrane. In the case of the β-subunit, three of the four tryptophan residues [28] are located in the hydrophilic regions. It seems, therefore, that after Mg^{2+} treatment the Trp-926 on the α-subunit (which is located near the membrane/water interface) and the Trp-31 on the β-subunit will emerge from the membrane and become accessible to KI. The Trp-100 residue, deeply buried in the hydrophobic interior, will remain in the membrane.

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References

1 Cantley, L.C. (1981) Curr. Top. Bioenerg. 11, 201–237
2 Swann, A.C. (1984) Arch. Biochem. Biophys. 233, 354–361
3 Amler, E., Teisinger, J., Svobodová, J. and Vyskočil, F., (1986) Biochim. Biophys. Acta 863, 18–22
4 Gupte, S.S., Lane, L.K., Johnson, J.D., Wallick, E.T. and Schwartz, A. (1979) J. Biol. Chem. 254, 5099–5103
5 Stahl, W.L. (1986) Neurochem. Int. 8, 449–476
6 Forgac, M. (1980) J. Biol. Chem. 255, 1547–1553
7 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
8 Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
9 Rephaeli, A., Richards, D. and Karlish, S.J.D. (1986) J. Biol. Chem. 261, 6248–6254
10 Karlish, S.J.D. and Pick, U. (1981) J. Physiol. (Lond.) 312, 505–529
11 Baba, A., Matsuda, T. and Iwata, H. (1977) Biochim. Biophys. Acta 482, 71–78
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Kuhry, J.G., Duportail, G., Bronner, C. and Laustriat, G. (1985) Biochim. Biophys. Acta 845, 60–67
14 Weber, G. (1977) J. Chem. Phys. 66, 4081–4091
15 Spencer, R.D. and Weber, G. (1970) J. Chem. Phys. 52, 1654–1663
16 Lakowicz, J., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508–519
17 Stern, I. and Volmer, M. (1919) Z. Phys. 20, 183–188
18 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263
19 Fullford, J. and Peal, C. (1980) Biochim. Biophys. Acta 598, 237–246
20 Esmann, M., Watts, A. and Marsh, D. (1985) Biochemistry 24, 1386–1393
21 Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695
22 Kawski, A. (1983) Photochem. Photobiol. 38(4), 487–508
23 Livingston, C.J. and Schachter, D. (1980) Biochemistry 19, 4823–4827
24 Marčelja, S. (1976) Biochim. Biophys. Acta 455, 1–7
25 Scott, H.L. (1986) Biochemistry 25, 6122–6126
26 Shinitzky, M. (1984) in Physiology of Membrane Fluidity (Shinitzky, M., ed.), pp. 17–22, CRC Press, Boca Raton, FL