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Effect of ouabain binding on the fluorescent properties of the Na^+/K^+-ATPase

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The influence of occupancy by ouabain of its specific binding site on the stability and conformation of the Na^+/K^+-ATPase has been investigated. When native Na^+/K^+-ATPase is exposed to guanidinium chloride or diluted acid, tryptophanyl fluorescence falls to 50% of the initial value. If ouabain is bound, higher concentrations of GdmCl or acidity are needed to reach the same decrease in fluorescence. The rotational diffusion coefficient (relaxation time), shows higher values for the Na^+/K^+-ATPase (ouabain) complex compared to the enzyme alone, suggesting an increase in molecular asymmetry. This observation is confirmed by the Stern-Volmer analysis that shows an increase in the accessibility of the fluorophores in the Na^+/K^+-ATPase (ouabain) ($K_{SV} = 15.6\ M^{-1}$) with respect to the native enzyme ($K_{SV} = 12.5\ M^{-1}$). Iodine perturbation of the enzyme labelled with FITC, demonstrates a decrease in the accessibility of the fluorescein probe in the Na^+/K^+-ATPase(ouabain) ($K_{SV} = 4\ M^{-1}$) compared to the Na^+/K^+-ATPase ($K_{SV} = 7\ M^{-1}$) indicating that after ouabain binding this site of the enzyme is less exposed to the solvent. These data, in agreement with other reports, suggest an allosteric effect of ouabain binding on the Na^+/K^+-ATPase conformation.

Introduction

Na^+/K^+-ATPase is a membrane bound enzyme which uses the energy of Mg-ATP binding and hydrolysis to regulate the sodium and potassium equilibrium of the cells [1,2].

The enzyme has been purified in a soluble form from several different animal species but despite the variety of isolation techniques and tissues used, all the purified enzymes are similarly composed by two subunits (α and β) [2–4]. The α subunit is a transmembrane polypeptide (100 kDa) containing both the ouabain and the ATP binding sites at the extracellular and intracellular side, respectively. The β subunit (50 kDa) is bound to the α subunit, and their dissociation causes inactivation of the enzyme.

The binding of cations to Na^+/K^+-ATPase stabilizes two different conformations of the en-

Abbreviations: Na^+/K^+-ATPase, sodium and potassium-dependent adenosine triphosphatase; Dns, dansyl (5-(dimethylamino)-1-naphthalenesulfonyl); Tris, tris(hydroxymethyl)aminomethane; GdmCl, guanidinium chloride; ATP, adenosine triphosphate; FITC, fluorescein isothiocyanate; $C_{12}E_8$, octaethylene glycol dodecyl monoether.

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zyme, the E_1 and the E_2 forms [1]. Na^+ ions compete at a single binding site and stabilize the E_1 form, while binding of K^+ ions promote the E_2 conformation: the E_1-E_2 transition is followed by an increase in tryptophanyl fluorescence [3,5].

Ouabain and other ligands are able [6] to produce fluorescence quenching of the extrinsic fluorescein fluorescent probe label of the E_2 conformation of Na^+/K^+-ATPase at the binding site. Karlish [5,7] has indeed demonstrated that fluorescein isothiocyanate covalently reacts with Na^+/K^+-ATPase thus leading to complete inhibition of the ATPase activity.

In the present work we have used both the intrinsic tryptophanyl and extrinsic fluorescein fluorescence change to show that ouabain enhances the resistance of Na^+/K^+-ATPase to both acid and guanidinium chloride inactivation. These observations suggest that ouabain binding to the Na^+/K^+-ATPase produces an alteration in the structure of the enzyme which presumably may be responsible for an increase in its stability.

Materials

Fluorescein isothiocyanate (FITC) and ouabain were obtained from Sigma. Guanidinium chloride (GdmCl) ultrapure grade, was purchased from Schwarz Mann. Chemicals not specifically mentioned were of reagent grade. Acrylamide was recrystallized from ethyl acetate.

Methods

Enzyme preparation. Na^+/K^+-ATPase was purified in membrane-bound form from the microsomal fraction of pig kidney outer medulla by selective solubilization with SDS of contaminating proteins [8]. The membrane-bound enzyme was then solubilized according to Brotherus et al. [9] using the detergent $C_{12}E_8$. After 1 h centrifugation at $105000 \times g$ the solubilized enzyme was collected from supernatant, concentrated on a Diaflo membrane under pressure of nitrogen and loaded on a Sephacryl P-300 Column. The purity of the enzyme was routinely tested on SDS-polyacrylamide gel electrophoresis as previously described [10].

Enzyme assay. The enzyme was assayed according to Anner et al. [11] after removal of $C_{12}E_8$ by incubation with Bio-Beads SM-2. Then 0.5–2.0 μg of the protein were added to 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 2.5 mM ATP-$Na_2H_2 \cdot 3H_2O$, 2 mM PEP, 1 mM Tris-EDTA, 25 mM imidazole (pH 7.2) plus pyruvate kinase/lactate dehydrogenase (PK/LDH) enzymes and NADH 82.88 mM. The absorbance decrease was read at 340 nm. The specific activity of our enzyme preparation was 1040 μmol P_i per mg protein per h at 37 °C.

Protein determinations were performed by the procedure described by Lowry et al. [12].

Preparation of fluorescent conjugate. Na^+/K^+-ATPase was conjugated with FITC in absence of ATP [13]. Enzyme was reacted at 1 mg protein per ml in 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA (pH 9.2) with 10 μM FITC for 30 min at 20 °C. The unbound FITC was removed by dialysis against 50 mM Tris-HCl (pH 7.5) for 24 h at 4 °C containing 1 mg/ml of bovine serum albumin. The degree of labelling varied between 0.8 and 1.2 mol/mol of protein. Ouabain was bound by the addition of 10^{-5} M ouabain to a medium containing 0.16 mg/ml of the enzyme, 5 mM $MgCl_2$, 0.1 M Tris-PO_4 (pH 7.4). Binding to the enzyme was routinarily measured using ^{3}H-ouabain in the same medium and corrected for non specific binding as described [14].

Ultraviolet fluorescence. Fluorescence spectra and intensities were obtained with a Perkin-Elmer 650-40 spectrofluorometer at 20 °C. Excitation and emission wavelengths were 280 and 340 nm for tryptophan and 495 and 525 nm for FITC.

Polarization of fluorescence. A Perkin-Elmer 650-40 spectrofluorometer was modified with a manual polarizing attachment (C.N. Wood Manufacturing, Newton, PA) to measure polarization of fluorescence. Excitation and emission wavelengths of tryptophan were set at 280 and 340 nm. Polarization (P) is defined as

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$$

where I is the intensity, $G = I_{hv}/I_{hh}$, and the first and second subscripts refer to the plane of polarization of the excitation and emission beams, respectively (v = vertical, h = horizontal).

The polarization data were analyzed by the Perrin equation:

$$(1/P) - (1/3) = (1/P_o - 1/3)(1 + 3\tau/\varrho)$$

where τ is the lifetime and ϱ the harmonic mean relaxation time.

Fluorescence lifetime measurement. Fluorescence lifetime of tryptophan chromophores were measured at 25°C, pH 8.0, with a nanosecond fluorescent lifetime apparatus.

Acrylamide and iodide quenching analysis. Acrylamide and iodide fluorescence quenching data were analyzed according to the Stern-Volmer equation

$$F_o/F = 1 + K_{SV}\,(Q)$$

where F_o and F are the tryptophanyl fluorescence intensities in the absence or presence of a quencher (Q) and K_{SV} is the dynamic quenching constant.

Statistical analysis. All the experiments were run in duplicate. Each point is the average of at least three different experiments. Standard deviation is not illustrated but the statistical comparison performed by the Student's *t*-test showed that the differences were statistically significant with $P < 0.01$.

Results

1. Stability

1a. Acid inactivation

The binding of ouabain affects the fluorescence properties of the extrinsic fluorescent probe FITC in the FITC modified enzymes [5], it seemed likely therefore that ouabain binding could also affect other physico-chemical parameters such as the acid lability of Na^+/K^+-ATPase.

So we measured the rates of the acid transition of Na^+/K^+-ATPase and of the complex Na^+/K^+-ATPase(ouabain) at three different pH values (Fig. 1). Lowering the pH (up to 4.0) resulted in increasing rates of fluorescence loss of Na^+/K^+-ATPase compared to Na^+/K^+-ATPase(ouabain). In particular, at pH 4.0 the fluorescence of Na^+/K^+-ATPase and Na^+/K^+-ATPase(ouabain) approached each other, suggesting that at this pH ouabain is probably released from its binding site and therefore has no influence on the final protein fluorescence.

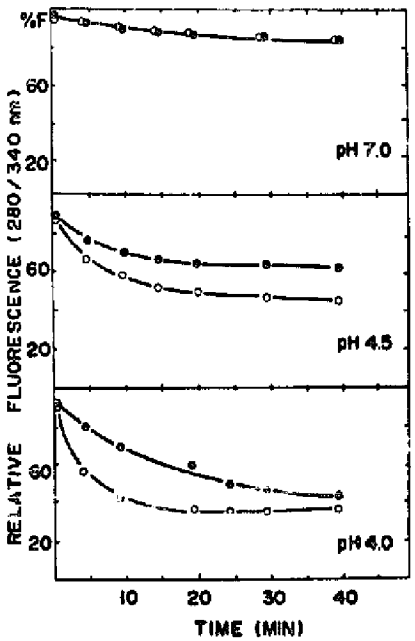

Fig. 1. Effect of pH on the rate of change of the tryptophanyl fluorescence of Na^+/K^+-ATPase (○) and Na^+/K^+-ATPase(ouabain) (●), 0.16 mg/ml in 0.1 M Tris-HCl, 5 mM $MgCl_2$ (pH 7.4), $T = 20$°C. The wavelengths of excitation and emission were 280 and 340 nm, respectively.

1b. GdmCl inactivation

As shown in Fig. 2, increasing concentrations of GdmCl from 0.5 M to 1.5 M resulted in increasing rates of fluorescence loss at pH 8.0 for Na^+/K^+-ATPase and Na^+/K^+-ATPase(ouabain). The time dependent fluorescence change observed in Na^+/K^+-ATPase (ouabain) was much lower than in the native enzyme. The binding of ouabain clearly has a significant effect in inhibiting the conformational transition induced by GdmCl.

2. Conformational effects

Considering that ouabain is able to enhance the stability of Na^+/K^+-ATPase towards acid and GdmCl exposure, it was important to determine whether the structure of the enzyme was modified by the ligand. We therefore evaluated the influence of ouabain on the rotational diffusion

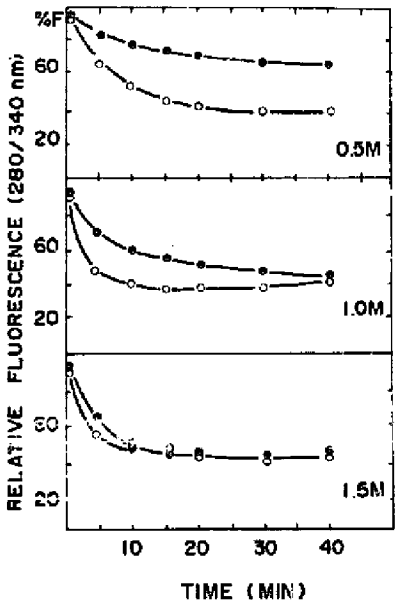

Fig. 2. Effect of GdmCl concentration on the rate of change of the tryptophanyl fluorescence of Na^+/K^+-ATPase (○) and Na^+/K^+-ATPase(ouabain) (●), 0.16 mg/ml in 0.1 M Tris-HCl, 5 mM $MgCl_2$ (pH 8.0), $T = 20\,^\circ C$. The wavelengths of excitation and emission were 280 and 340 nm, respectively.

coefficient (i.e. relaxation time) of the Na^+/K^+-ATPase. The relaxation time was evaluated from the dependence of the polarization of tryptophanyl fluorescence on the viscosity of the solution.

A liftime of 1 ns was found at 20 °C for the tryptophanyl residues of Na^+/K^+-ATPase. The lifetime of tryptophanyl chromophores are normally too small to be useful for the application of the Perrin equation to proteins of the size of Na^+/K^+-ATPase. However, a significant increase in the tryptophanyl polarization was observed with ouabain addition. The increase in tryptophanyl polarization of Na^+/K^+-ATPase and Na^+/K^+-ATPase(ouabain) after addition of sucrose is plotted in Fig. 3. Relaxation times (ϱ) of 12.5 ns and 14 ns at 20 °C were calculated from the data shown in Fig. 3 for Na^+/K^+-ATPase and Na^+/K^+-ATPase(ouabain), respectively. The higher relaxation time of Na^+/K^+-ATPase(ouabain) suggests that ouabain binding results in a decrease in rotational flexibility of domains containing the tryptophanyl residues.

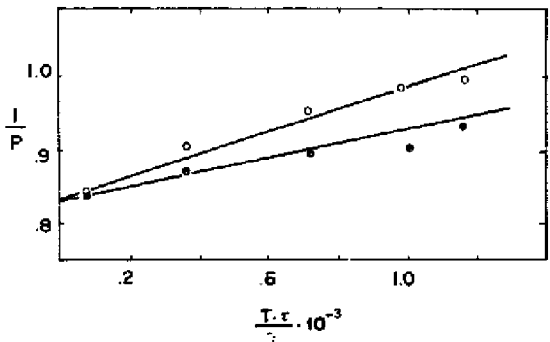

Fig. 3. Perrin plots of the polarization of the tryptophanyl fluorescence of Na^+/K^+-ATPase (○) and Na^+/K^+-ATPase(ouabain) (●) 0.04 mg/ml in Tris-HCl, 5 mM $MgCl_2$ (pH 7.4), $T = 20\,^\circ C$. The sucrose concentration varied from 0 to 60%. The wavelengths of excitation and emission were 280 and 340 nm, respectively.

3. *Tryptophanyl heterogeneity*

The heterogeneity of tryptophanyl emission from multitryptophanyl containing proteins has been demonstrated in several ways including lifetime measurements [15,16], quenching by collisional quenchers, bound ligands [17–20] and by circular polarized emission [21]. We have used the

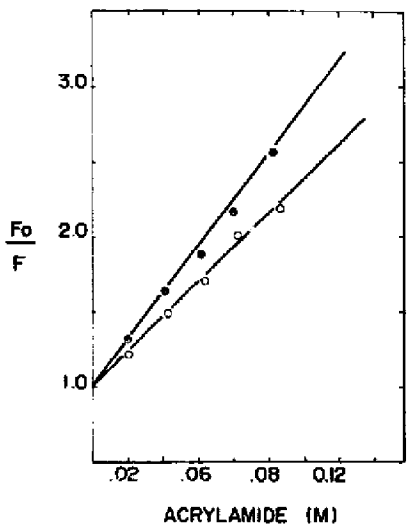

Fig. 4. Quenching of fluorescence (F) of Na^+/K^+-ATPase (○) and Na^+/K^+-ATPase(ouabain) (●) by 0.04 mg/ml acrylamide, 0.1 M Tris-HCl, 5 mM $MgCl_2$ (pH 7.4), $T = 20\,^\circ C$. F_o is the fluorescence intensity in the absence of acrylamide. The wavelength of excitation and emission were 280 and 340 nm, respectively.

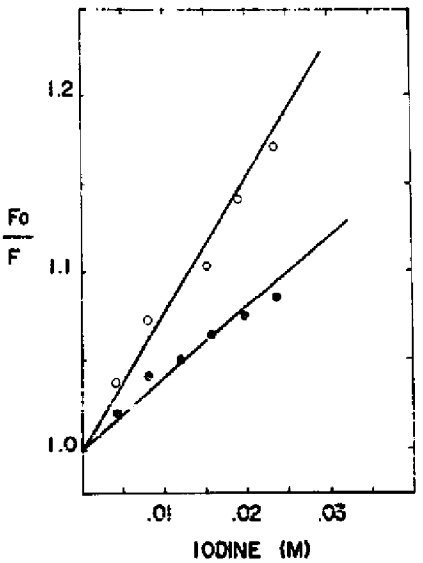

Fig. 5. Quenching of fluorescence (F) by iodine of Na^+/K^+-ATPase/FITC (○) and Na^+/K^+-ATPase/FITC (ouabain) (●) 0.04 mg/ml. Conditions as in Fig. 4. F_o is the fluorescence intensity in the absence of iodine. The wavelengths of excitation and emission were 495 and 525 nm, respectively.

uncharged collisional quencher acrylamide not only to show tryptophanyl heterogeneity but also to detect a conformational change produced by ouabain binding.

3a. Stern-Volmer analysis of acrylamide quenching

The relative accessibility of the different tryptophanyl residues to the solvent can be measured by the concentration dependence of acrylamide quenching [22]. A K_{SV} of 12.5 M^{-1} (Fig. 4) was found for the Na^+/K^+-ATPase excited at 280 nm. The K_{SV} value when ouabain is bound, increases from 12.5 M^{-1} to 15.6 M^{-1}. Thus a larger number of groups remain accessible to acrylamide when ouabain is bound.

3b. Stern-Volmer analysis of iodide quenching of Na^+/K^+-ATPase/FITC

The slopes for Stern-Volmer quenching by I^- were determined for Na^+/K^+-ATPase/FITC and Na^+/K^+-ATPase/FITC(ouabain). The value is 7 M^{-1} for the Na^+/K^+-ATPase/FITC and 4 M^{-1} in the presence of ouabain (Fig. 5) indicating that the FITC is located in a protected environment.

Discussion

The binding of small ligands can enhance the stability of proteins. When avidin for instance, binds biotin a large increase in the thermal transition temperature from 85 to 132°C is generated [23]. The binding of thyroxin to TBG, a serum protein devolved to the transport of thyroid hormones, also produces conformational changes which are responsible for the increase in resistance of the complexed protein to denaturation [24].

Two major conformations of the Na^+/K^+-ATPase have been identified, E_1 and E_2. The E_1 form binds Na^+ or K^+, but Na^+ is bound with a greater affinity. If K^+ is bound, a conformational transitional change takes place and the species generated is the enzyme in the E_2 form. In this form the enzyme has also been demonstrated to be less thermolabile [25,26]. The conditions that promote ouabain binding are such that the preferred ouabain binding species is the E_2 conformation [27]. The transitions between the E_1 and E_2 forms of the protein are accompanied by rearrangements of a significant number of residues in the α subunit [28] and by an increase in the susceptibility of the same peptide bond to protease [29,30]. Structural changes may also involve sulphydryl groups [31]. Our finding that Na^+/K^+-ATPase(ouabain) is more resistant than the native enzyme to inactivation by either acid or GdmCl, may lead to attribute the increase in stability to a structural reorganization consequent to ouabain binding. The various methods employed clearly show that conformational changes occurring after ouabain binding may explain the variations in the degree of exposure of tryptophan residues to the solvent. Also the rotational correlation time of the soluble preparation of the whole enzyme containing at least one α and one β subunit should be expected greater than the 12.5 to 14 ns reported in our experiments. Hydrodynamic studies have reported to rule out the extreme asymmetry which would be required to give such small relaxation times. Therefore polarization values should reflect segmental freedom of the protein domains containing the tryptophanyl chromophore.

Also the experiments with acrylamide agree with the increase in relaxation time, since the increase in collisional quenching rate constant in

the absence of static quenching suggests that the tryptophan residues are more exposed to the solvent in Na^+/K^+-ATPase(ouabain) than in Na^+/K^+-ATPase. Finally, the effect of ouabain binding to the Na^+/K^+-ATPase/FITC on iodine quenching, compared to the Na^+/K^+-ATPase/FITC alone, showed a decrease in the accessibility of the FITC bound. This observation suggests that ouabain binding to the E_1 conformation of the enzyme stabilizes the protein generating a E_2 form characterized by a decrease in the degree of the exposure to the solvent of the microenvironment at the FITC binding site.

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References

1 Jørgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68.
2 Wallick, R.T., Lane, L.K. and Schwarz, A. (1979) Annu. Rev. Physiol. 41, 397–341.
3 Maczydlawski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2357–2366.
4 Craig, W.S. and Kyte, J. (1980) J. Biol. Chem. 255, 6262–6269.
5 Karlish, S.J.D. (1979) in Na, K-ATPase. Structure and Kinetics (Skou, J.C. and Norby, J.G. eds.), pp. 115–118, Academic Press, New York.
6 Karlish, S.J.D. (1980) J. Bioenerg. Biomembr. 12, 111–136.
7 Karlish, S.J.D., Beaugé, L.A. and Glynn, I.M. (1979) Nature 282, 333–335.
8 Jørgensen, P.L. (1974) Methods Enzymol. 32 B, 277–290.
9 Brotherus, J.R., Jacobsen, L. and Jørgensen, P.L. (1983) Biochim. Biophys. Acta 731, 290–303.
10 Grimaldi, S., Pozzi, D., Pascale, E., D'Onofrio, M., Verna, R., Giganti, G., Monaco, F. and Roche, J. (1987) C.R. Soc. Biol. 181, 609–615.
11 Anner, B.M., Marcus, M.M. and Moosmayer, M. (1984) Enzymes, Receptors and Carriers of Biological Membranes, (Azzi et al., eds.), pp. 81–96, Springer-Verlag, Berlin, Heidelberg.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
13 Carilli, C.T. (1982) J. Biol. Chem. 257, 10, 5601–5606.
14 Hegyvary, C., Chigurupati, R., Kang, K. and Mahoney, D. (1980) J. Biol. Chem. 255, 3068–3074.
15 Shaklai, N., Gafni, A. and Daniel, E. (1978) Biochemistry 17, 4438–4442.
16 Gafni, A. and Weber, M. (1979) Arch. Biochem. Biophys. 196, 363–370.
17 Lehrer, S.S. (1967) Biochem. Biophys. Res. Commun. 13, 5170–5178.
18 Grinwald, A. and Steinberg, I.Z. (1974) Biochemistry 13, 5170–5178.
19 Secemsky, I., Lehrer, S.S. and Lienhard, G. (1972) J. Biol. Chem. 247, 4740–4748.
20 Pellet, R. and Edelhoch, H. (1973) J. Biol. Chem. 248, 5443–5447.
21 Vuk Pavlovic, S., Isenman, D.E., Elgavish, G.A., Gafni, A., Licht, A. and Pecht, I. (1979) Biochemistry 15, 672–680.
22 Eftink, M.R. and Ghiron, C.A. (1976) Biochemistry 15, 672–680.
23 Donovan, J.W. and Ross, K. (1973) Biochemistry 21, 145–151.
24 Grimaldi, S., Edelhoch, H. and Robbins, J. (1982) Biochemistry 21, 145–151.
25 Fisher, T.H. (1983) Biochem. J. 211, 771–774.
26 Jørgensen, P.L. and Andersen, J.P. (1986) Biochemistry 25, 2889–2897.
27 Hegyvary, C. and Jørgensen, P.L. (1981) J. Biol. Chem. 256, 6296–6303.
28 Jørgensen, P.L., Skriver, E., Hebert, H. and Maunsbach, A.B. (1982) Ann. N.Y. Acad. Sci. 402, 203–219.
29 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97–108.
30 Castro, J. and Farley, R.A. (1979) J. Biol. Chem. 254, 2221–2228.
31 Jørgensen, P.L. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 597, 305–317.