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Effect of divalent cations on Na^+,K^+-ATPase obtained from human placenta

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Circular dichroism (CD) and acrylamide quenching studies of Na^+,K^+-ATPase from human placenta showed that its incorporation into phosphatidylcholine vesicles increased the enzymic activity by 55%. Moreover, both with the purified and the vesicle-reconstituted protein, Ca^{2+} and Mg^{2+} increased the activity, the effect being more pronounced after preincubation of the protein with Mg^{2+}. CD data suggest that this activity increase may be linked to a change in the secondary structure of the ATPase, in particular β-turn, β-sheet and random coil. Acrylamide quenching studies suggest that ions could primarily interact with phospholipid head groups, but not directly with the protein.

Na^+,K^+-ATPase; Human placenta; Fluorescence; Quenching; Circular dichroism

1. INTRODUCTION

The addition of divalent cations, such as Ca^{2+} and Mg^{2+}, to Na^+,K^+-ATPase obtained from different sources, can modify both the enzyme and its physicochemical properties [1–4]. In fact, Na^+,K^+-ATPase requires Mg^{2+} for full activity while the action of Ca^{2+} may be either stimulatory or inhibitory, probably depending upon the origin of the enzyme [5].

It is generally assumed that the enzyme passes through different conformational changes during its transport cycle [6] and that phospholipids (PL's) are necessary for the actvity [6].

Recent reports suggested that 2 mM Mg^{2+} may act primarily on phospholipid head groups, and that the subsequent change in lipid order can be due to protein arrangement [4]. Ordering effects by millimolar concentrations of Ca^{2+} were observed in microvillus plasma membrane obtained from human placenta [7]. In the same membrane, Na^+,K^+-ATPase activity was increased by the addition of millimolar concentrations of Mg^{2+} [8] or Ca^{2+} (unpublished results from this laboratory).

Even the purified Na^+,K^+-ATPase from human placenta shows a positive modulation of its activity by similar concentrations of Ca^{2+} [9] and Mg^{2+} [3]. Purified Na^+,K^+-ATPase activity is preserved in the presence of phospholipid and cholesterol molecules surrounding the protein [6,10], however, ATPase activity is greatly increased upon incorporation into lipid vesicles, although the degree of recovered activity depends on the lipid composition in the proteoliposome [11]. The reason for this recovery of activity is not clear, although some hypotheses have been suggested [11]. Moreover, a detailed understanding of the protein–lipid interaction is still lacking. In the present work, CD and acrylamide quenching studies were done to elucidate the effect of incorporation into egg phosphatidylcholine vesicles on the protein structure. In addition, the effect of millimolar concentrations of Ca^{2+} and Mg^{2+} on the protein structure and activity was studied both with purified and reconstituted placental Na^+,K^+-ATPase.

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2. MATERIALS AND METHODS

Egg phosphatidylcholine (EPC) was provided by Avanti Polar (Alabaster, AL, USA). Commercial acrylamide was recrystallized from ethyl acetate.

2.1. *Purification of Na^+,K^+-ATPase*

The enzyme was isolated by a modification of the method of Jorgensen [12]. The preparation showed two major bands (112 kDa and 44 kDa) on SDS-gel electrophorsis corresponding to the two subunits of Na^+,K^+-ATPase. The protein was then washed in double-distilled water and lyophilized.

2.2. *Reconstitution experiments*

The isolated enzyme was reconstituted in EPC vesicles according to Kasahara and Hinkle [13]. Briefly EPC (2.8 mg in 2.3 ml of buffer) was sonicated for 30 min at 20°C under nitrogen. Freeze-dried protein (0.5 mg) was added to 1.3 ml of sonicated liposomes. The mixture was quickly frozen in a dry-ice/ethanol bath and thawed at room temperature for 5 min, followed by a brief sonication of 25 s. Vesicles obtained by this preparation were unilamellar of 200–2,000 Å diameter, as reported by Kasahara and Hinkle [13]. The determination of the Na^+,K^+-ATPase activity was done according to the method of Kitao and Hattori [14]. The results were tested for statistical significance by Student's *t*-test. To check the effect of divalent cations on protein

activity, the ATPase was preincubated with the appropriate ion concentration for 1 h.

2.3. *Circular dichroism (CD)*

CD spectra were recorded on a Jasco spectropolarimeter under constant nitrogen flux at 20°C. The path length was 1 mm and the protein concentration was 90 μg/ml in Tris-HCl buffer (40 mM, pH 7.4). The experiments were performed both on purified and reconstituted protein. When present, the EPC vesicles were 0.34 mM Readings were done against a reference cuvette containing the same components except the protein. Secondary structure estimation was done according to Chang et al. [15].

2.4. *Fluorescence measurements*

The tryptophan fluorescence quenching data were obtained by adding increasing concentrations of an 8 M solution of acrylamide, and corrected for acrylamide absorbance [16]. Fluorescence was recorded in a Perkin-Elmer MPF 66 spectrofluorimeter at an excitation wavelength of 295 nm. The final protein concentration was 90 μg/ml. Intensity was corrected for dilution. The experiments were performed both on purified and reconstituted protein. When present, the vesicles were 0.34 mM.

3. RESULTS

The specific activity of purified Na^+,K^+-ATPase was 315 μmol Pi per mg protein per h, while EPC incorporation resulted in an activity of 490 μmol Pi per mg protein per h, thus an increase of 55%. The relatively low activity shown by the enzyme isolated from placenta plasma membranes is in disagreement with our previous work [9], but in the present work we used a protein that was isolated and then lyophilized. Lyophilization could modify its structural characteristics, as suggested by CD results, and hence its activity. Table I shows the effect of divalent cations on purified and reconstituted ATPase. Data at lower concentrations of divalent cations were not reported because they were not significant. 2 mM Mg^{2+} did not modify enzyme activity which was slightly increased by the same Ca^{2+} concentration. After incubation with 1 mM or 2 mM Mg^{2+}, Ca^{2+} significantly increased enzyme activity at both concentrations tested. This effect is evident both on purified and reconstituted ATPase. Moreover, concentrations higher than 2 mM did not produce a further increase in the enzymatic activity (data not shown). CD data obtained for Na^+,K^+-ATPase purified from human placenta are shown in Table II. The secondary structure of the protein was greatly affected by the presence of Mg^{2+} ions in the medium. Under these conditions the results are substantially in agreement with those obtained by IR measurements [17,18]. Moreover, CD data showed that incorporation into EPC vesicles modifies the β-sheet (from 47.5% to 21%), the β-turn (from 17% to 29%) and the random coil (from 20.5% to 34.5%), but not the α-helical content (15–15.5%) of the protein. Both purified and EPC-reconstituted ATPase showed a similar change in secondary structure, although with different numerical values, when both 1 mM Ca^{2+} and 1 mM Mg^{2+} were present in the solution. In fact, in each case, the protein structure showed an increase of β-turn and random coil and a decrease of β-sheet (Table II). The secondary structure of the protein was unaffected by 1 mM Ca^{2+} (Table II). Acrylamide quenching plots are reported in Fig. 1. Using the equation reported in [4] and the assumption used in that work, a K_q (Stern–Volmer constant) and f_a (fractional maximum accessible protein fluorescence) were calculated for both purified and EPC-reconstituted protein (Fig. 1). Briefly, it is assumed that all Trp residues present in the protein are independent, equally absorbing, fluorophores under conditions of validity of the Stern–Volmer law for each fluorophore [19]. Moreover, it is supposed that all Trp residues, buried in the hydrophobic part of the membrane, are totally inaccessible to acrylamide, and that the accessibility of the Trp is described by the same Stern–Volmer constant, K_q. In this way the Stern–Volmer equation becomes [20]:

$$F_0/(F_0 - F) = (1/f_a)(1/K_q[Q] + 1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the concentration of the quencher and f_a is the fractional maximum accessible protein fluorescence. The data obtained suggest that the incorporation into EPC vesicles results in a lower Stern–Volmer constant (K_q) (decrease from 3.83 to 2.14) and in a decrease of accessibility of Trp residues to acrylamide (by some 20% residues exposed to acrylamide quenching). In both

Table I

Na^+,K^+-ATPase activity in isolated (A) and EPC-reconstituted (B) enzyme in the presence of Ca^{2+} and/or Mg^{2+}

Mg^{2+} (mM)	Ca^{2+} (mM)	A	% A	B	% B
–	–	315.00 ± 1.80	–	490.01 ± 3.60	–
1	–	315.40 ± 2.40	–	491.03 ± 5.00	–
–	1	316.43 ± 1.80	–	494.01 ± 5.80	–
2	–	325.40 ± 3.00*	+ 3.3	507.15 ± 2.50*	+ 3 5
–	2	328.23 ± 1.50**	+ 4.3	525.28 ± 3 50**	+ 7 2
1	1	348.71 ± 2.70**	+10.7	662.97 ± 5.40**	+35.3
2	2	414.54 ± 2.80**	+31 8	621.32 ± 6 60**	+26 8

The results are expressed as μmol Pi · mg^{-1} · h^{-1} and as a percentage of the increase with respect to control activity without cations Data represent the mean of three different experiments ± S.D. *$P < 0.05$, **$P < 0.01$, as calculated by Student's t-test.

cases no modifications are caused by the presence of the divalent cations (Fig. 1).

4. DISCUSSION

Previous studies showed that ATPase activity is increased on incorporation into phospholipid vesicles or by interaction with divalent cations, such as Ca^{2+} and Mg^{2+}. In the present work the protein from human placenta and lyophilized was incorporated into EPC vesicles and studied in the absence and presence of divalent cations. Na^+,K^+-ATPase consists of two subunits, α and β, both integral membrane proteins. The oligomeric organization of the protein is uncertain: it may exist as $\alpha\beta$ or $(\alpha\beta)_2$ [11]. Moreover, the domain for cation binding has not yet been determined, although recent studies suggest the involvement of two negatively charged residues in a non-aqueous environment [21]. Ca^{2+} and Mg^{2+} are known to modulate ATPase activity by an unknown mechanism. Previous studies suggested that these cations can interact primarily with phospholipid head groups [22], but recent reports on ATPase purified from pig kidney outer medullar [4] suggested that 2 mM Mg^{2+} induced structural modifications in the lipid surrounding the protein. These structural modifications bring about a more stable protein conformation and a greater exposure of Trp residues to quenching with KI [4]. Previous studies showed an increase in microviscosity, as probed by ESR, induced by 1 mM Ca^{2+} on microvillus membranes of human placenta [7]. Moreover, this Ca^{2+} concentration causes a clustering of intramembrane particles (usually associated with integral membrane proteins) in the same membranes [7]. The protein used in this work has been highly purified, as demonstrated by the SDS-gel electrophoresis pattern, showing only two bands of the α and β subunits (data not shown). Our data demonstrated that incorporation of placental Na^+,K^+-ATPase into EPC vesicles greatly increases its activity (see section 3) as well as a modification in the secondary structure of the protein. The increase in activity may be linked to a change in the secondary structure but not necessarily in the α-helical content.

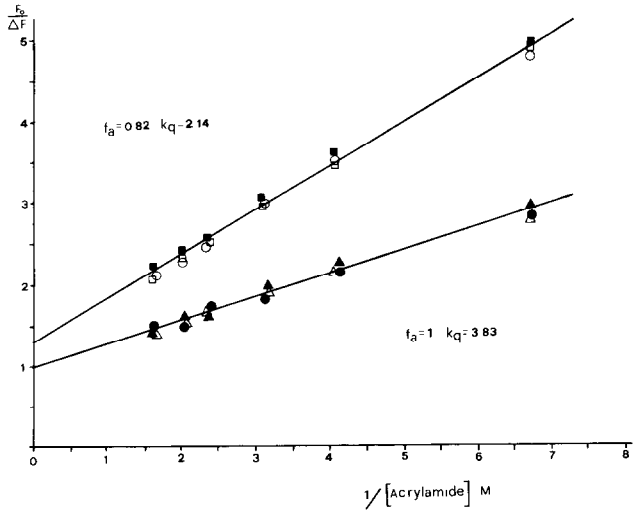

Fig. 1. Modified Stern–Volmer plots for the quenching of the fluorescence of purified and reconstituted Na^+,K^+-ATPase in the presence or absence of divalent cations. Purified ATPase: without ions (△); with 1 mM Ca^{2+} (▲); with 1 mM Ca^{2+} and 1 mM Mg^{2+} (●). EPC reconstituted protein: without ions (□), with 1 mM Ca^{2+} (■); with 1 mM Ca^{2+} and 1 mM Mg^{2+} (○).

Present data showed that acrylamide, which selectively quenches surface Trp residues, has less access to Trp after incorporation of the enzyme into vesicles, while no effect due to the presence of ions was detectable. This could be due to the interaction with microenvironments different from the Trp ones. Since both α and β subunits contain some Trp residues [23], these results could be in agreement with the hypothesis that ions could primarily interact with phospholipids, however, a change in the physical state of phospholipids could be the cause of the detected changes in the structure of the protein, as suggested by Amler et al. [4].

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Table II

Percentage of α-helix, β-sheet, β-turn and random coil conformations in isolated and EPC-reconstituted Na^+,K^+-ATPase

Mg^{2+} (mM)	Ca^{2+} (mM)	α-Helix	β-Sheet	β-Turn	Random coil
Isolated Na^+,K^+-ATPase					
–	–	15	47.5	17	20.5
–	1	14.5	49	17.5	19
1	1	15	29.5	25.5	30
EPC-reconstituted Na^+,K^+-ATPase					
–	–	15.5	21	29	34.5
–	1	15	24	28	33
1	1	13.5	14.5	32.5	39.5

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