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Dynamic lipid–protein stoichiometry on E_1 and E_2 conformations of the Na^+/K^+-ATPase ☆

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ABSTRACT

Annular lipid–protein stoichiometry in native pig kidney Na^+/K^+-ATPase preparation was studied by [^{125}I]TID-PC/16 labeling. Our data indicate that the transmembrane domain of the Na^+/K^+-ATPase in the E_1 state is less exposed to the lipids than in E_2, i.e. the conformational transitions are accompanied by changes in the number of annular lipids but not in the affinity of these lipids for the protein. The lipid–protein stoichiometry was 23 ± 2 (α subunit) and 5.0 ± 0.4 (β subunit) in the E_1 conformation and 32 ± 2 (α subunit) and 7 ± 1 (β subunit) in the E_2 conformation.

1. Introduction

The reaction cycle of the of all P-type ATPases involves transition between two conformational states, E_1 and E_2. As most membrane proteins, their function is affected by the structures of lipid molecules that surround them in the membrane [1]. These solvent lipids have been referred to as boundary or annular lipids to denote the fact that they form an annular shell around the protein. The purpose of the present work was to analyze the changes in the annular lipids under conditions that stabilized the E_1 or E_2 conformations and to estimate the lipid–protein stoichiometry. Na^+/K^+-ATPase, a well-characterized representative of the P-ATPase superfamily, served as an object of this investigation.

Na^+/K^+-ATPase consists of the catalytic α subunit, a glycosylated β subunit and in most tissues this pump is associated with a small protein of the FXYD family, the γ subunit. The α subunit contains three cytoplasmic domains and 10 transmembrane helices (M1–M10) while the β- and γ-subunits each cross the phospholipids bilayer only once [2].

Interactions between the Na^+/K^+-ATPase and its surrounding lipids were studied by Esmann and Marsh [3] using electron spin resonance (ESR) and by Blanton et al. [4] employing highly non-polar reagents that partition into the hydrophobic core of the membrane. To the best of our knowledge, however, none of the previous studies addressed the correlation between lipid–protein stoichiometry and conformational states in Na^+/K^+-ATPase.

We have used [^{125}I]TID-PC/16, an analogue of phosphatidylcholine provided with a photoactivatable group at the end of one of the fatty acyl chains [5–7], to measure the number of annular lipids in the native preparation of the Na^+/K^+-ATPase. Our results show significant differences between [^{125}I]TID-PC/16 labeling of the E_1 and E_2 states.

2. Materials and methods

2.1. Enzyme and reaction conditions

Na^+/K^+-ATPase was partially purified from pig kidney according to Klodos et al. [8]. Our preparation consists predominantly of Na^+/K^+-ATPase (see also Fig. 1 in Ref. [8]), with nucleotide binding capacity of 2.5 nmol/mg protein and ATPase activity of 25 μmol Pi (min × mg protein)$^{-1}$. Incubations were performed at 25 °C in

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media containing 25 mM imidazole–HCl (pH 7.4 at 25 °C) and 0.25 mM EDTA (buffer medium).

2.2. [^{125}I]TID-PC/16 labeling procedure

[^{125}I]TID-PC/16 was prepared as already described by Villamil et al. [5]. Since this probe was used in trace amounts (<40 nM, i.e. less than 1/1000 of the total amphiphiles), the possibility of cross-linking between [^{125}I]TID-PC/16 and other [^{125}I]TID-PC/16 already tethered to the enzyme is negligible.

2.3. Membrane-bound Na^+/K^+-ATPase

A dried film of the photoactivatable reagent was resuspended in $C_{12}E_{10}$ and incubated with membrane-bound protein during 20 min. These conditions (100 μg protein/ml, 0.0001% $C_{12}E_{10}$) are known to allow the [^{125}I]TID-PC/16 to partition in the phospholipid milieu without solubilization of the membrane protein. Then the particular conformation of the enzyme was induced by incubation with buffer alone or plus 150 mM of choline chloride, NaCl, or KCl (20 min), or 1 mM ouabain (40 min). Protein final concentration was 50 μg/ml. After 15 min of photolysis (with light from a filtered UV source, $\lambda = 360$ nm) the [^{125}I]TID-PC/16 reacts indiscriminately with its surrounding molecular cage [7]. This time was selected from the time course of photolysis, which followed a single increasing exponential function ($t_{1/2} \approx 2$ min), and shows that the specific incorporation is constant from 10 to at least 25 min. The samples were precipitated with trichloroacetic acid (TCA) and subjected to electrophoresis according to the Tris–Tricine SDS–PAGE method [9]. The α- and β-subunit bands stained with Coomassie Blue R (see Fig. 1) were excised from the gel, and the ^{125}I-radioactivity was measured in a gamma counter. Detection of the γ subunit (7.5 kDa) in our experimental conditions is not possible since it migrates together with the running front in the acrylamide gel. Protein amount was also measured in each stained band after elution [10]. The dialysis step, previously used to wash out the excess reagent [5], was replaced by several washings with TCA. That improved the yield of labeled enzyme and reduced the blanks.

2.4. Solubilized Na^+/K^+-ATPase

The labeling procedure was essentially the same as for the membrane-bound protein, except that the enzyme was initially solubilized with 0.5% $C_{12}E_{10}$ during 15 min at 4 °C. After centrifugation the supernatant containing Na^+/K^+-ATPase was exposed to the [^{125}I]TID-PC/16 resuspended in $C_{12}E_{10}$. The samples were then incubated with either buffer alone or plus 150 mM choline chloride, NaCl, or KCl for 20 min. Final concentration was 50 μg protein/ml. From this point, the process was the same as that used for the membrane-bound Na^+/K^+-ATPase.

Since it is not possible to accurately measure the recovery of [^{125}I]TID-PC/16 after the radioiodination procedure, we are not able to precisely calculate the specific activity of the probe. For that reason, we expressed the results as cpm/mol of protein. Both for membrane-bound and solubilized enzyme, the background radioactivity and optical density measured per unit area of the gel were less than 3% of that containing the protein band.

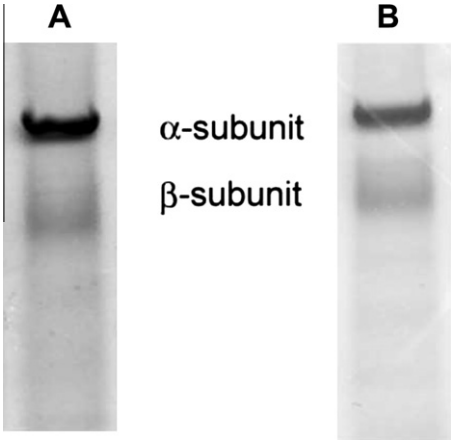

Fig. 1. SDS–PAGE of membrane-bound (A) and solubilized Na^+/K^+-ATPase (B) after labeling and photolysis.

2.5. Lipid–protein stoichiometry

We employed the solubilized enzyme preparation. $C_{12}E_{10}$ micelles containing the protein and [^{125}I]TID-PC/16 were mixed with increasing amounts of dimyristoyl phosphatidylcholine (DMPC) in the presence of 150 mM of KCl or NaCl. $C_{12}E_{10}$ concentration was constant (0.075%) for all conditions. Micelles of DMPC/$C_{12}E_{10}$ were sonicated before mixture with the enzyme.

2.6. Stability of the enzyme

The loss of ATPase activity of membrane-bound and solubilized enzyme after labeling and photolysis was less than 5% and 10%, respectively.

2.7. Equilibrium fluorescence experiments

The membrane-bound Na^+/K^+-ATPase (50 μg/ml final concentration) was incubated under equilibrium conditions in reaction media with eosin 0.4 μM alone or plus 150 mM of choline chloride, NaCl, KCl, or 1 mM ouabain. Eosin fluorescence was measured in an Aminco-Bowman/Series 2 spectrofluorimeter at 25 °C, with 520 nm excitation wavelength and a 550 nm cut-off filter on the emission side.

2.8. Data analysis

The equations were fitted to the experimental data by a non-linear regression procedure based on the Gauss–Newton algorithm using commercial software (Excel™ 7.0 and Sigma-Plot™ 6.0 for Windows™).

3. Results

3.1. Quantitative [^{125}I]TID-PC/16 labeling of E_1 and E_2 states of membrane-bound Na^+/K^+-ATPase

The membrane-bound Na^+/K^+-ATPase preparation was equilibrated with [^{125}I]TID-PC/16 and then incubated in reaction media containing no added ligands, choline chloride, NaCl, KCl, or ouabain. The radioactivity and protein concentration corresponding to the α and β subunits were measured to calculate the level of covalently bound [^{125}I]TID-PC/16. In Na^+-containing media the specific incorporation was 3.6 and 1.0×10^{12} cpm/mol protein for the α and β subunits, respectively. Fig. 2 shows, both for the α and β subunits, that the incorporation of [^{125}I]TID-PC/16 in K^+ or ouabain-containing media is higher than in buffer alone or in the presence of Na^+ or choline. The amount of the covalently attached probe to the K^+-bound E_2 conformation is 1.4-fold higher compared to the Na^+-bound E_1, suggesting significant differences in their surfaces exposed to the lipid phase. Note that the binding of [^{125}I]TID-PC/16 to the α subunit was always about 3.5-fold higher than that to the β subunit (panel C, Fig. 2).

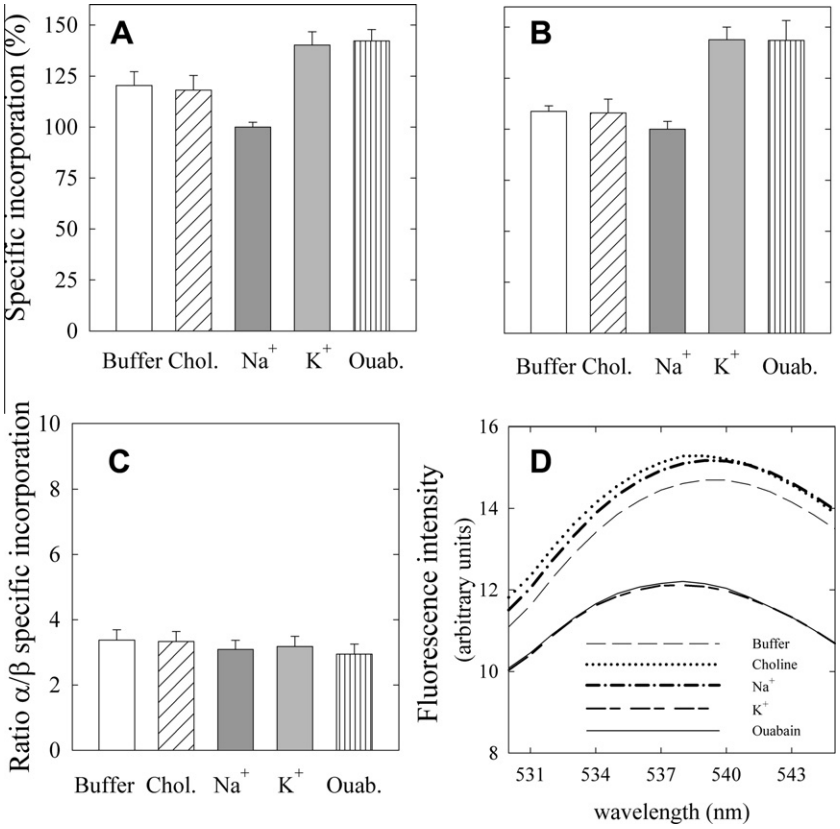

Fig. 2. Covalent labeling of the α (panel A) and β (panel B) subunits of Na^+/K^+-ATPase in buffer medium (buffer) or plus 150 mM of either choline chloride (Chol.), NaCl, KCl, or 1 mM ouabain (Ouab.). All data are expressed in percent of the value obtained in Na^+-containing medium. Panel C: ratio of incorporation between α and β subunits. Panel D: emission spectra of eosin fluorescence.

To confirm that the applied ligands do induce the expected conformational states of the enzyme, eosin fluorescence was measured under identical conditions (but omitting [^{125}I]TID-PC/16). As expected [11,12], higher fluorescence levels were obtained for the enzyme incubated with Na^+ or choline, which induce the E_1 conformation, as compared to those obtained in media with K^+ or ouabain, which induce the E_2 conformation.

3.2. Quantitative [^{125}I]TID-PC/16 labeling of the solubilized Na^+/K^+-ATPase in the E_1 and E_2 states

The Na^+/K^+-ATPase solubilized in $C_{12}E_{10}$ and mixed with [^{125}I]TID-PC/16 was diluted in media containing no added ligands, choline chloride, NaCl, or KCl. In the presence of NaCl the radioactivity incorporation reached 1.7×10^{13} cpm/mol α subunit vs. 5.6×10^{12} cpm/mol β subunit. The results (Fig. 3) are essentially the same as those obtained with the membrane-bound Na^+/K^+-ATPase suggesting that the solubilization neither modifies the distribution of the probe between transmembrane helices of the α and β subunits nor significantly changes the mutual arrangement of the transmembrane helices. Nevertheless, the relative effect of K^+ vs. Na^+ on the labeled amount was slightly higher in solubilized than in membrane-bound Na^+/K^+-ATPase.

3.3. Lipid–protein stoichiometry

We used solubilized enzyme to quantify the number of annular lipids. Fig. 4 shows the displacement of [^{125}I]TID-PC/16 incorporation for the α and β subunits with increasing DMPC concentrations in the presence of 150 mM Na^+ or K^+.

Considering that [^{125}I]TID-PC/16 and DMPC bind to the Na^+/K^+-ATPase as equivalent molecules [3,5] one can describe the matrix of binding equilibria between protein and phospholipids by the Adair equation for equivalent and independent sites. In accordance, the radioactivity measured per mol of subunit decreased as a hyperbolic function of DMPC concentration: $Y = Y_{Max} \times K_{0.5}/([DMPC] + K_{0.5})$. To calculate the number of lipid molecules in direct contact with the protein (N), the data from Fig. 4 were transformed into mol of DMPC by dividing the specific incorporation (radioactivity bound per mol of enzyme) by the specific activity calculated for each [DMPC] [5]. The insets in Fig. 4 show that N increases with the concentration of DMPC along a hyperbolic function, $N = N_{Max} \times [DMPC]/(K_{0.5} + [DMPC])$, where N_{Max} and $K_{0.5}$ are the lipid–protein stoichiometry and the apparent dissociation constant, respectively. Table 1 shows the values of the parameters describing the best fits in the insets of Fig. 4 for the α and β subunits (continuous lines). In the K^+-containing media (E_2) the α subunit binds about 32 molecules of DMPC and this value decreases to 23 for E_1 state. For the same change in conformation the lipid-protein stoichiometry for the β subunit decreases from 7 to 5.

4. Discussion

This paper shows that [^{125}I]TID-PC/16 is a suitable probe to quantify the number of annular lipids for the α and β subunits of the Na^+/K^+-ATPase and to detect movements in the intramembrane lipid-exposed segments upon E_1–E_2 transition.

Blanton and McCardy [4] working with *Torpedo* Na^+/K^+-ATPase found an enhanced photoincorporation of [^{125}I]TID but not of [^{125}I]TID-PC/16 to the transmembrane segments of the α subunit in E_2 with respect to E_1. More recently, Villamil et al. [5] used [^{125}I]TID-PC/16 to determine the lipid–protein stoichiometry of the Na^+/K^+-ATPase in a single condition. In the present work, we demonstrate that labeling of the enzyme with [^{125}I]TID-PC/16 var-

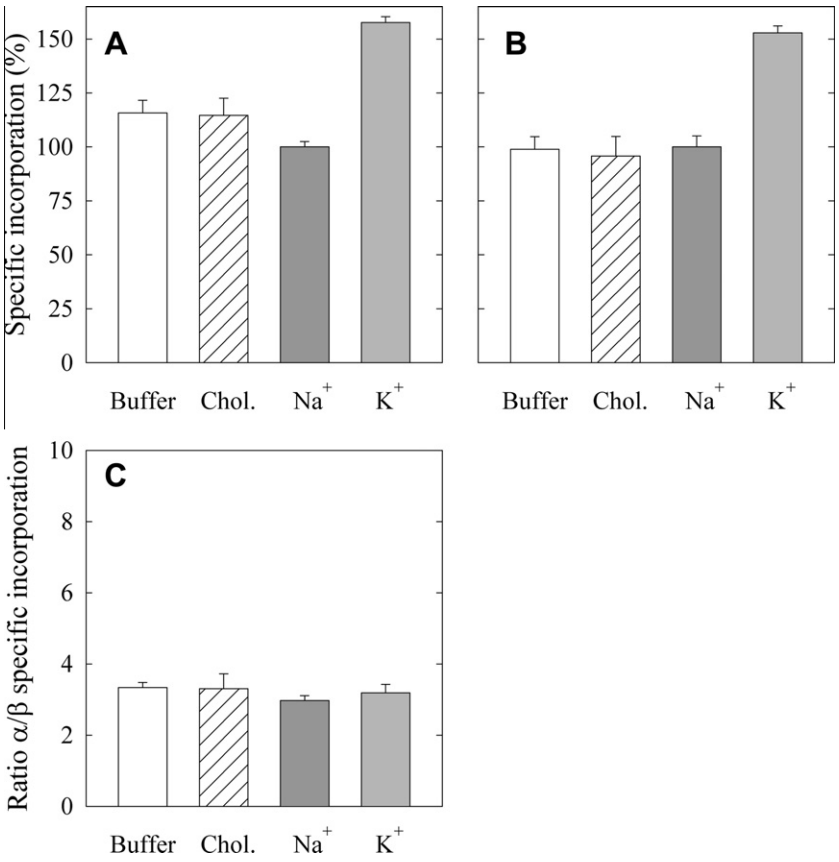

Fig. 3. Covalent labeling of α (panel A) and β (panel B) subunits of the solubilized Na^+/K^+-ATPase incubated in buffer medium (buffer) or plus 150 mM of either choline chloride (Chol.), NaCl or KCl. All data are expressed in percent of the value obtained in Na^+-containing medium. Panel C: ratio of incorporation between α and β subunits.

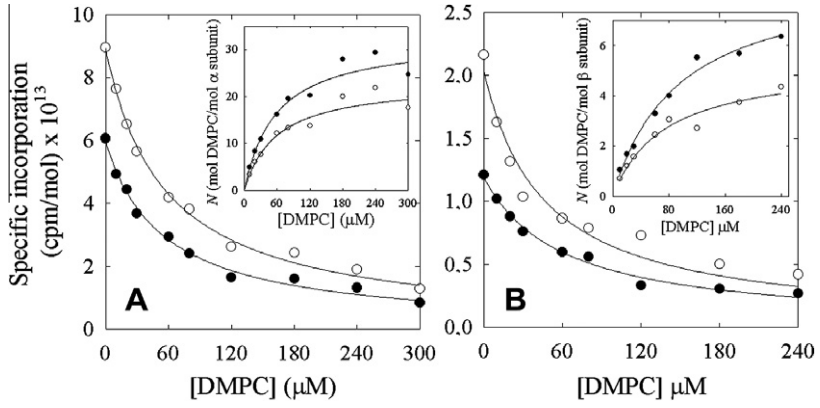

Fig. 4. Specific labeling with [^{125}I]TID-PC/16 of the Na^+/K^+-ATPase reconstituted in $C_{12}E_{10}$/DMPC micelles as a function of [DMPC] for the α (panel A) and β (panel B) subunits, in the presence of 150 mM NaCl (○) or KCl (●). Inset shows *N* as a function of [DMPC].

ies with the enzyme conformation, as found by Mangialavori et al. for PMCA and SERCA [6]. The higher labeling in E_2 observed for both the α and β subunits seems to be due to a change in the number of annular lipids but not in the affinity of these lipids for the protein (Table 1). The enzyme solubilized in $C_{12}E_{10}$ shows essentially the same behavior as the membrane-bound enzyme although the conformation-induced difference in labeling is somewhat higher for the solubilized preparation. Since E_2 is known for its higher tendency to form oligomers than E_1 [13], the oligomerization of the solubilized protein is not an explanation for this result.

Table 1
The best fitting values (average ± S.E.) of the parameters of the curves of *N* as a function of [DMPC] used to fit the data shown in the insets of Fig. 4.

		N_{Max} (mol DMPC/mol protein subunit)	$K_{0.5}$ (μM)
K^+ media	α Subunit	32 ± 2	52 ± 3
	β Subunit	7 ± 1	63 ± 13
Na^+ media	α Subunit	23 ± 2	53 ± 5
	β Subunit	5.0 ± 0.4	62 ± 7

The fact that changes in photolabeling of the enzyme correspond to changes in eosin fluorescence can be taken as evidence of the coupling between movements in the transmembrane segments and in the nucleotide binding site.

Our data on stoichiometry are in general agreement with the number of lipids per αβ protomer obtained for kidney

(31.5 ± 1.5) or shark (33 ± 3) Na^+/K^+-ATPase by ESR [3]. The value of 32 ± 2 found in this study for α subunit in E_2 is however higher than the 24 ± 5 lipid sites per α reported by Villamil Giraldo et al. [5]. Possibly, the improved washing procedure and a wider range of DMPC concentrations tested allowed us a closer approach to saturation.

The observed ratio of 4.5 between the number of annular lipids surrounding α and β subunits does not contradict the known structure of the Na^+/K^+-ATPase in the E_2 conformation [14]. Thus, the transmembrane domain of the α-subunit (10 helices) can be represented by a cylinder with a diameter of 45 Å, while the single helix of the β-subunit is a cylinder with a 10 Å diameter. The surface of the β-subunit is partially shielded due to the interaction with α-subunit, but its tilted position with respect to the membrane increases the surface of protein–lipid contacts.

The absolute number of the annular lipids surrounding the β subunit (5–7) also seems reasonable. Assuming that a lipid in the crystalline bilayer occupies a surface of 70 $Å^2$ the effective diameter of a lipid molecule is 9.4 Å [15] so between 4 and 9 phospholipids (depending on whether it contacts the protein with both or only one chain) could be placed around the β subunit (10 Å diameter). We have not estimated the number of annular lipids surrounding the γ-subunit. Data from the literature, however, allow some approximation. The 10-lipids difference in the number of annular lipids between Na^+/K^+-ATPase and Ca^{2+}-ATPase reported by Esmann and Marsh [3] might be due to the heterooligomeric structure of the Na^+/K^+-ATPase. According to our results, the β-subunit makes contact with 5–7 lipid molecules depending on the conformation. The residual molecules (5–3) could be ascribed to the γ-subunit, suggesting that a single peripheral transmembrane helix (here exemplified by either β or γ) has contact with 5 lipids on average.

The extent of the conformational transition necessary to induce the observed change in the number of annular lipids does not have to be immense. A loosening/compaction of the multi-helix structure and/or tilt changes of the helices can explain our results. Regarding tilt, this could modify the cylindrical cross section of the helices, e.g. from circular to elliptical, sufficiently to explain the observed changes in the number of annular lipids. If these modifications were concerted between the α and β subunits, it would not be surprising that the ratio between the numbers of lipids tethered to the α and β subunits was the same (as we found) regardless of the conformational state.

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