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Investigation of reconstitution of the Na,K-ATPase in lipid vesicles

Yu. A. Ivashchuk-Kienbaum, R. Riek and H.-J. Apell

Department of Biology, University of Konstanz, Konstanz (Germany)

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Vesicles containing Na,K-ATPase were prepared by a dialysis method in buffers with various concentrations of K^+ and Na^+ ions. Ion-exchange chromatography has been used to separate proteoliposomes into protein-depleted and protein-rich fractions. The pumping activity of reconstituted ion pumps has been determined in the different fractions of the vesicle preparation using voltage-dependent fluorescence dyes. This method allowed to characterise vesicle fractions by a quantity which is proportional to the average number of pumps per vesicle with an active (inside-out) orientation. It could be shown that both, the amount of enzymatic active protein and the orientation of Na,K-ATPase in the vesicle lipid bilayer, is partially controlled by the Na^+ and K^+ concentration in the buffer during vesicle formation. High Na^+ concentrations preferentially maintain the E_1 conformation of the enzyme, which is less stable against denaturation during the dialysis, but displays a higher percentage of inside-out orientation of the transport-active protein. High K^+ concentrations maintain the E_2 conformation of the enzyme, which is stable against denaturation during the dialysis, but leads to a random orientation of the pump during dialysis.

Introduction

Na,K-ATPase is an integral plasma membrane protein that utilises the free energy of ATP hydrolysis to generate per pumping cycle a coupled efflux of 3 Na^+ and influx of 2 K^+ ions, both against their electrochemical gradient [1–8]. The enzyme consists of two subunits, the α-subunit and the β-subunit whose molecular masses (approx. 112 kDa and 35 kDa, respectively) are known from the amino-acid sequence [9,10]. Na,K-ATPase contains an equal number of sites for phosphorylation, high-affinity ATP binding, ouabain binding and vanadate binding [6]. In its normal mode of operation, Na,K-ATPase performs a cycle of conformational transitions, ion-binding and ion-release steps. Transport functions, which are vectorial processes, can be investigated only in a compartmentalized system in which two compartments are separated by a membrane that restricts exchange of ions on the action of the transport protein. To study the ion-transport mechanism and the kinetics of conformational transitions, a powerful approach has been developed by functional reconstitution of ATPase into the membrane of closed phospholipid vesicles. Since Jørgensen in 1974 has described methods to isolate Na,K-ATPase from different tissues, several methods have been published to reconstitute the protein [11–18]. To investigate the electrogenicity of Na,K-ATPase different methods were used: fluorescence-labelled Na,K-ATPase which shows a fluorescence quenching upon the conformational transitions, can be examined by stopped-flow techniques [19,20], membrane potentials are maintained by potassium concentration gradients and the potassium ionophore valinomycin [21]. Another method is the detection of ^{22}Na uptake in proteoliposomes at different membrane potentials [22,23] where membrane potentials have been maintained by ion gradients and ionophores. A third method is the direct use of potential-sensitive fluorescence dye Oxonol VI to measure pumping activity [24–26].

The use of vesicles with reconstituted ion pumps has the advantage that only the protein of interest is investigated and that experiments are highly reproducible. But a main problem in the quantitative analysis of experiments with proteoliposomes is the inhomogeneity of the number of pumps per vesicle and their orientation. There are three possible orientations of the Na,K-ATPase in the vesicle: right-side out, which is

Correspondence to: H.-J. Apell, Department of Biology, University of Konstanz, Universitätsstraße 10, 7750 Konstanz, Germany.

the native orientation in the cell membrane with cytoplasmic aspect inward, inside-out, with cytoplasmic aspect outward, and Na,K-ATPase that is unreconstituted having both cytoplasmic and extracellular side exposed, referred to as non-oriented [24]. Several functional tests have been applied to test the sidedness of the protein: (a) ouabain sensitivity of catalytic activity before and after vesicle opening and (b) vanadate binding. It has been suggested that the sidedness of the reconstituted protein depends on the protein-lipid ratio [27]. The preparation method used in this paper results in 50% inside-out orientation of the reconstituted ATPase [17]. From electron micrographs of freeze-fractured vesicles it has been estimated that protein-containing vesicles have incorporated different numbers of protein molecules [28]. Therefore, a quantitative interpretation of the results related to the functional features of the protein must include a statistical analysis of the protein distribution, of the average density of the protein molecules which have a functional (inside-out) orientation and of the vesicle size [28].

Vesicle preparation containing Na,K-ATPase can be characterized by various parameters: radius of vesicles and distribution of radii, pump orientation and distribution, lipid-protein ratio, internal volume of vesicles and pump activity. It has been shown, that the size of vesicles is controlled by a Gaussian distribution and the number of pumps by a Poisson statistics [16]. Using the dialysis method described in Ref. 16, the average diameter of ATPase-containing vesicles obtained by dynamic light-scattering was 96 nm with a half-width of 10 nm. These findings made clear that most of the parameters used to describe vesicle preparations are only mean values averaged over the whole ranges of radii and number of pumps. Since in the quantitative analysis of experiments with reconstituted vesicles those averaged parameters are used, the resulting numbers can be misleading. Therefore, the control of these vesicle parameters is important.

The aim of this work is to study factors that influence the reconstitution of Na,K-ATPase in lipid vesicles and to describe conditions which allow to get well defined fractions of vesicles with the help of ion-exchange chromatography.

Materials and Methods

Materials

Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL), Sodium dodecylsulfate (SDS) from Pierce (Rockford, IL) and sodium cholate from Merck (Darmstadt, Germany). Phospho-*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, sonderqualität) were from Boehringer-Mannheim (Mannheim, Germany). 5-Iodoacetamidofluorescein (5-IAF) and Oxonol VI (bis(3-propyl-5-oxoisoxazol-4yl)pentamethinoxonol) were from Molecular Probes (Junction City, OR). The dialysis tube (pore radius 2.4 nm) was purchased from Serva (Heidelberg, Germany). Q-Sepharose (strong anion exchanger) was purchased from Pharmacia. Ion-exchange separation was performed with HPLC System, Model 590 Programmable Solvent Delivery Module, from Millipore Waters Chromatography Division (Danvers, MA).

Buffers. Buffer H: If not otherwise indicated, the buffer for the vesicle experiments contained 30 mM imidazole, 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM $MgSO_4$, the pH was adjusted to 7.2 with H_2SO_4.

Buffer A: 180 mM glycerine, 15 mM imidazole and 5 mM K_2SO_4, the pH was adjusted to 7.2 with H_2SO_4.

Buffer B: 200 mM K_2SO_4, 15 mM imidazole, the pH was adjusted to 7.2 with H_2SO_4.

Methods

Enzyme preparation and fluorescence labelling. Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen [29]. This method yields a purified enzyme in the form of membrane fragments containing about 0.6 mg phospholipid and 0.2 mg cholesterol per 1 mg protein [29]. The used preparations had a specific activity in the range between 1600 and 2200 μmol P_i/h per mg protein at 37°C, corresponding to a turnover rate of 120–170 s^{-1} (based on a molar mass of 280000 g/mol). The Na,K-ATPase-rich membrane fragments (about 3 mg protein per 1 ml) were suspended in a buffer of 25 mM imidazole, 1 mM EDTA, 10 mg/ml saccharose (pH 7.5) and frozen in samples of 300 μl. In this form the preparation could be stored for several months at −70°C without significant loss of activity.

Fluorescence labelling of the enzyme with 5-IAF was performed according to Stürmer et al. [30] by incubating 200–300 μg of the enzyme for 24 h at 4°C with a solution containing 200 μM 5-IAF, 10 mM K_2SO_4 and 50 mM imidazole (pH 7.5). The labelled enzyme was concentrated and separated from unbound dye molecules by centrifugation for 15 min at 100000 $\times g$ in a Beckman airfuge at 20°C.

Vesicle preparation. The Na,K-ATPase vesicles were prepared according to a previously published method [15,16]. 2 mg of the enzyme were solubilized in 1 ml 'cholate buffer', consisting of buffer H with 1% sodium cholate and different K^+/Na^+ ratios: 70 mM K_2SO_4 and 5 mM Na_2SO_4 (preparation 1), 25 mM K_2SO_4 and 50 mM Na_2SO_4 (preparation 2), 5 mM K_2SO_4 and 70 mM Na_2SO_4 (preparation 3), and 0 mM K_2SO_4 and 150 mM Na_2SO_4 (preparation 4). Equal volumes of a lipid/detergent solution [16] and the enzyme solubilizate were mixed, corresponding to a protein/lipid

ratio of about 0.7 mg protein per 7 mg lipid (1 : 10). 200 μl of the combined solution were transferred to a 6 mm dialysis tubing and dialysed for 72 h at 4°C against 200 ml buffer H containing K_2SO_4 and Na_2SO_4 in appropriate concentration used in the vesicle preparation procedure. Pure lipid vesicles were prepared from dioleoylphosphatidylcholine by the same procedure, except for the addition of protein.

Analytical procedures. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [31]. The enzymatic activity, E_A, of reconstituted protein has been determined at 37°C according to Ref. 24 in the absence and presence of the detergent $C_{12}E_8$. Vesicles formed with the method described in the previous paragraph become extremely leaky above 25°C when added to the assay buffer. This observation was manifested by the finding that the specific enzymatic activity measured with untreated vesicles and vesicles 'reopened' by $C_{12}E_8$ was almost the same (with an averaged difference of about 2%). Thus, the given numbers of E_A, as noted in the Results section, represent the enzymatic activity of the total reconstituted proteins.

Protein concentrations have been determined by the Lowry method [32] using bovine serum albumin as a standard. The concentration of dioleoylphosphatidylcholine in micelle and liposome suspensions were determined by the enzymatic phospholipid B test [33].

Fluorescence measurements. Fluorescence experiments were carried out in a Perkin-Elmer 650-40 fluorescence spectrophotometer as has been described in Ref. 34. If not indicated otherwise, the excitation wavelength was set to 580 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 20 nm). All measurements were performed at 7.1 ± 0.2°C. The other experimental conditions for the measurement of the vesicles transport activity were the same as in Ref. 34.

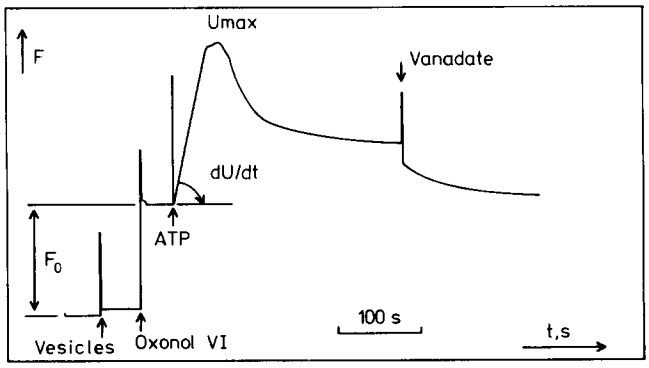

Fig. 1. Membrane potential generated by Na,K-ATPase reconstituted in the lipid vesicles. The initial rate of voltage change, dU_m/dt, was calculated from the initial slope of $\Delta F/F_0$ versus time t, using an independently determined fluorescence-voltage calibration [25]. Buffer composition was buffer H with 140 mM Na^+ + 10 mM K^+. 70 nM Oxonol VI, 5 mM ATP and 20 mM vanadate were added successively to the vesicle containing solution. The temperature was 7.2°C.

After the addition of ATP to the external medium, inside-out oriented pump molecules became activated and fluorescence changes were observed corresponding to inside-positive potentials of up to 150–300 mV. From the initial slope of the fluorescence signal, $d/dt(\Delta F/F_0)$, as shown in Fig. 1, the initial rate of voltage change can be estimated using the independently determined fluorescence-voltage calibration [25]. Addition of sodium ortho-vanadate to a final concentration of 20 mM to the buffer caused an instantaneous inhibition of the Na,K-ATPase. In Fig. 1 the effect of vanadate can be seen by a drop of the fluorescence amplitude, which is an artefact, and a following exponential decay indicating the decreasing membrane potential. It has been shown, that the time constant of the exponential decay, τ, is defined by the expression C_m/Λ_m [26]. To calculate the initial potential change we have used a partition coefficient for Oxonol VI, γ, of 19000.

Ion-exchange chromatography. The column (10 × 1 cm) was packed with 6 ml of suspension of the anion-exchanger, Q-Sepharose, washed thoroughly and equilibrated with buffer A. 100–200 μl of the vesicle solution was injected and eluted successively with buffer A (containing 5 mM K_2SO_4) and buffer B (containing 200 mM K_2SO_4) at a flow rate of 1 ml/min. The two fractions of vesicles from buffer A and B were separately collected. All procedures were carried out at room temperature. One half of each vesicle containing fractions (unseparated, A and B) was used to determine immediately the ATP-induced initial pumping rate by the fluorescence method described above. The other half of each fraction was transferred into a dialysis tube and dialysed for 48 h at 4°C against 200 ml buffer H with the same K_2SO_4 and Na_2SO_4 concentration as used during vesicle formation (see above). This was done in order to achieve the same osmotic conditions as before the chromatography (the unseparated fraction was included as a reference). After dialysis the ATP-induced initial pumping rate was determined again for all three fractions.

Part of the vesicle preparation could not be removed even by the maximum salt concentration used for elution. To remove this tightly bound material the column was flushed with buffer B containing in addition 1% $C_{12}E_8$. Due to the action of the detergent no vesicular structures could be maintained in the eluate.

Results

Chromatographic separation of vesicles containing reconstituted Na,K-ATPase

To fractionate vesicles containing reconstituted Na,K-ATPase into more homogeneous subpopulations

different approaches can be used. Density centrifugation had turned out to be too insensitive to separate vesicles sufficiently in terms of diameter or number of pumps [39]. Affinity chromatography with covalently bound ATP allowed the isolation of different classes of vesicles, but the production of column material which was active with Na,K-ATPase (ATP-agarose, Type 4 from Pharmacia) is no longer available. Using anion-exchange chromatography with Q-Sepharose gave promising results which are presented in the following.

The active group of Q-Sepharose is a quaternary amine ($-CH_2-N^{\oplus}(CH_3)_3$). It interacts with negative charges of the Na,K-ATPase. Pure lipid vesicles are eluded with the void volume (Fig. 2). To trace proteoliposomes in the eluate the protein has been labelled with 5-IAF and its fluorescence has been detected by a HPLC fluorescence monitor (Shimadzu, RF 353). In the case of protein-free vesicles light scattering of the solution has been detected. Testing vesicle preparations with different protein/lipid ratios between 0.02 and 0.5 (w/w), it has been demonstrated that the salt concentrations necessary to remove the bulk part of vesicles from the ion exchanger depends on the protein content (Fig. 2). In these experiments the ionic strength in the buffer has been increased in steps of 10 mM K_2SO_4. A continuous salt gradient resulted in comparable results (data not shown). To keep the osmotic strength constant the salt has been replaced by appropriate concentrations of glycerine in buffer A. Separating vesicles with low protein/lipid ratios (≤ 0.1) it has been shown that part of the enzymatic and transport-active vesicles was eluded with buffer A (5 mM K_2SO_4). To test the interaction between proteoliposomes and ion-exchanger matrix, vesicles with different protein

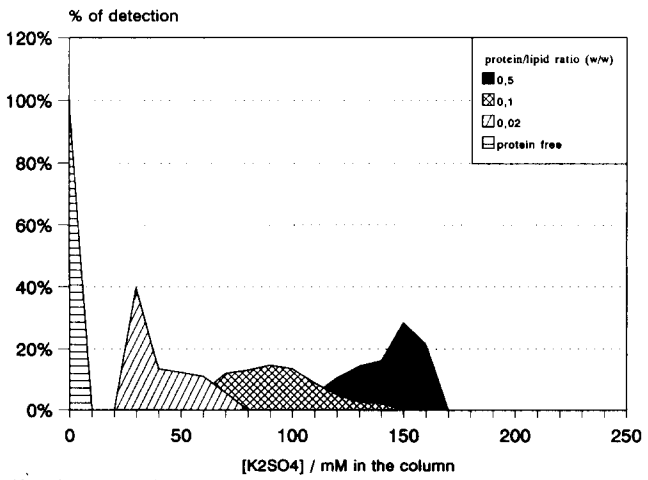

Fig. 2. Ion-exchange separation of vesicles with various protein content. Separation was performed in a buffer containing 180 mM glycerine, 15 mM imidazole (pH 7.2) and a salt gradient from 5 mM K_2SO_4 to 200 mM. The temperature was 4°C. Flow rate 1 ml/min. Samples of 200 μl vesicle suspension were injected. The protein content was maintained by the corresponding protein/lipid ratio at the vesicle preparation procedure.

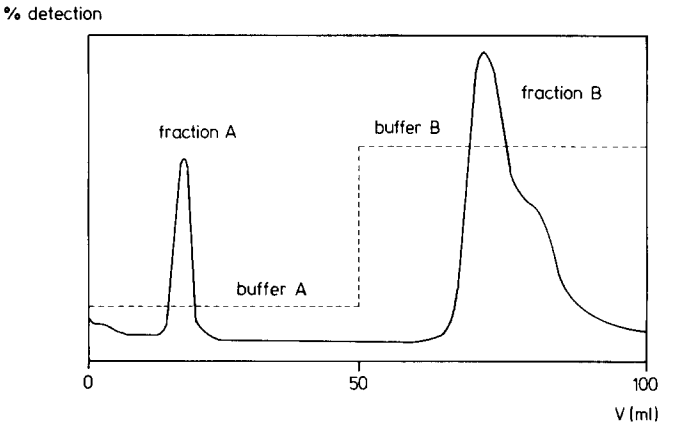

Fig. 3. Ion-exchange separation of the vesicles with reconstituted Na,K-ATPase. Fraction A, low-protein vesicles; fraction B, high-protein vesicles. Buffer A, 180 mM glycerine, 15 mM imidazole, 5 mM K_2SO_4 (pH 7.2); buffer B, 200 mM K_2SO_4, 15 mM imidazole (pH 7.2) Separation was performed at 4°C with a flow rate of 1 ml/min. Samples of 200 μl vesicle suspension were injected.

content have been separated. The results are shown in Fig. 2. They led to the suggestion that the amount reconstituted protein determines the retardation of the elution and, therefore, anion-exchange chromatography with Q-Sepharose can be used as a method to distinguish between vesicles with different numbers of protein molecules per vesicle. At the moment it is not possible to determine how the interaction of the ion exchanger and the two different faces of the protein occurs. The cytoplasmic face of the protein is a bulky part of the protein, it carries approx. 40% of the protein mass and includes the ATP-binding site. The extracellular face is much smaller (approx. 20% of the protein mass), but is glycosylated with a series of polar sugars.

Applying a continuous salt gradient in the chromatography produced highly-diluted samples of vesicles, which had to be concentrated to determine lipid contents, transport or enzymatic activity. Therefore, we decided to perform a separation in two steps (buffer A and B) and to collect probes at the elution peaks in small volumes of high vesicle concentrations. The standard vesicle preparations had a protein content of 0.1 mg per mg lipid. In these preparation the portion of transport-incompetent, protein-free vesicles was negligible [28]. The eluation pattern of such a preparation is shown in Fig. 3. Both fractions, A and B, have been collected separately for further investigations.

The first step of analysis was the determination of lipid and protein contents of both fractions and their comparison with the unseparated preparation. As can be seen from Fig. 4A a considerable amount, about 30% of the total lipid and 50–60% of the protein of the preparation, remained bound to the Q-Sepharose of the column. Routinely the quantity of bound lipid and protein has been determined as the difference

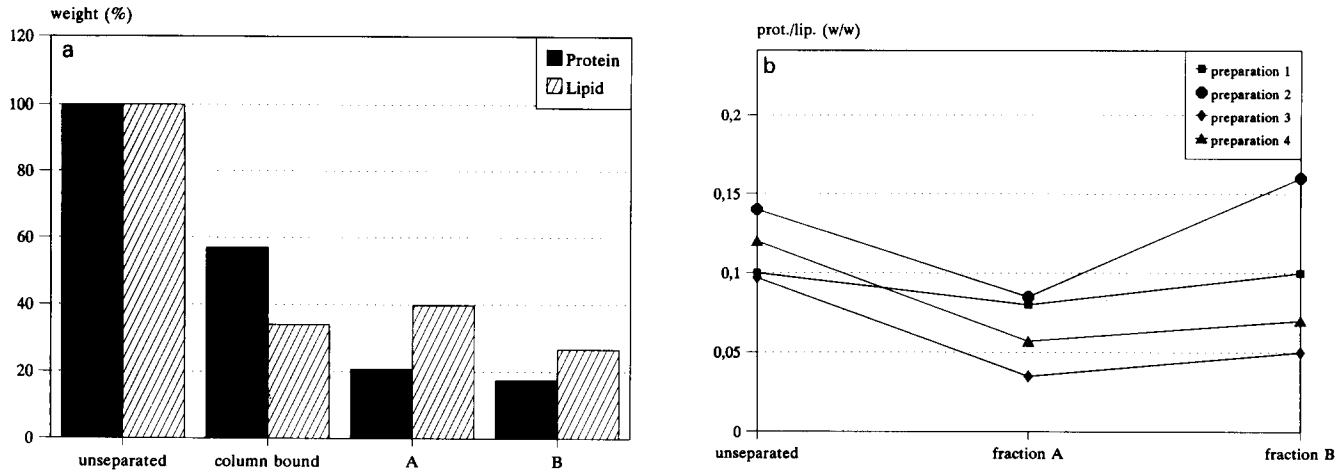

Fig. 4. (a) Averaged protein and lipid content in per cent of unseparated vesicles and after the separation (fractions A and B) Protein concentrations have been determined by the Lowry method [32] using bovine serum albumin as a standard. The concentration of dioleoylphosphatidylcholine was determined by the enzymatic phospholipid B test [33]. Column-bound protein and lipid was determined as difference between injected and collected material. Fraction A was eluted with buffer A (180 mM glycerine, 15 mM imidazole, 5 mM K_2SO_4 (pH 7.2)), fraction B with buffer B (200 mM K_2SO_4, 15 mM imidazole, (pH 7.2)) Temperature was 4°C, flow rate 1 ml/min. Samples of 200 μl vesicle suspension were injected. (b) Relative protein content of the vesicles before and after the separation for all vesicle preparations. Buffer compositions were: Preparation 1, 140 mM $K^+ + 10$ mM Na^+; preparation 2, 50 mM $K^+ + 100$ mM Na^+; preparation 3, 10 mM $K^+ + 140$ mM Na^+; preparation 4, 0 mM $K^+ + 150$ mM Na^+. Other conditions as in part (a) The corresponding ratio of protein/lipid of the column bound material was 1.2 (preparation 1), 1.9 (2), 2.05 (3) and 2.2 (4).

between injected and eluted material, since it was impossible to elute this portion without detergent. To compare these fractions we have characterised them by their protein/lipid ratio, which is an indirect measure of the average protein content per vesicle. As expected the relative content of protein was smaller in fraction A than in fraction B (Fig. 4B). The protein/lipid ratio in both fractions A and B was smaller than in the unseparated preparation. This finding can be explained only by the assumption that protein-rich structures remained bound to the column. As shown in Fig. 4A, approx. 50% of the total protein was not eluted by a salt concentration of 200 mM. Since this protein could be removed from the column only by detergent-containing buffers, the eluate contained only mixed micelles of detergent, lipid and protein and it could not be determined, whether the protein was reconstituted in vesicles previously. Instead of reconstitution in vesicles the protein could still be maintained in open membrane fragments, comparable to the purified microsomal preparation. When the protein is solubilized by the cholate treatment in buffers with high K^+ concentrations during the vesicle preparation, the sedimentation step does not lead to a completely rigid pellet and the removal of the supernatant could easily contain fragments or larger protein/lipid aggregates. Both, membrane fragments or vesicles with patches pump aggregates, are expected to bind tightly to the ion-exchanger material.

Analysis of the electrogenic pump activity

A second set of parameters to characterise a vesicle preparation can be obtained by analysis of the electrogenic pump activity. It is assumed that all inside-out reconstituted pumps work with a comparable turnover rate when activated by ATP. The biological membrane can be described physically by two parameters, the specific membrane capacitance, C_m, and (leak) membrane conductance, Λ_m. The change of the membrane potential, dU_m/dt, combines these parameters and electrogenic pumping activity by following relation:

$$dU_m/dt = (n_p e_o v/AC_m - \Lambda_m U_m/C_m) \quad (1)$$

where n_p is the number of inside-out (functionally) oriented pump molecules per vesicle, e_o the elementary charge, v the turnover rate of the pump and A the average area of the vesicle membrane [25]. Adding ATP to the extravesicular medium, only those pumps become activated which face the outside with their ATP-binding site ('inside-out orientation'). Since the Na,K-ATPase transports 3 Na^+ ions outward and 2 K^+ ions inward, one net elementary charge is transported per cycle and the pumps generate an inside-positive membrane potential across the vesicle membrane [25]. From these experiments the initial increase and the maximum value of the membrane potential have been analysed.

The voltage-induced fluorescence change, ΔF, is induced by a voltage-dependent redistribution of Oxonol VI between water and vesicle membrane. Since the magnitude of ΔF depends critically on the lipid concentration in these experiments [25], we have determined the lipid concentration for each vesicle sample

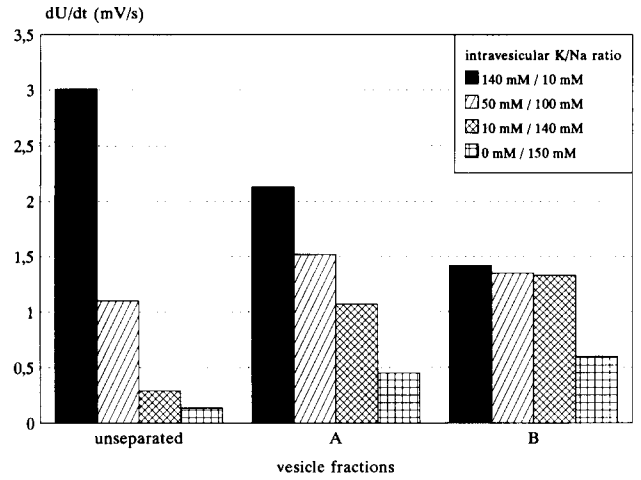

Fig. 5. The initial rate of voltage change, dU/dt, of vesicles before and after the ion-exchange chromatography. Vesicles were prepared in buffer H with various K^+/Na^+ ratio. The initial rate has been estimated from the initial slope of $\Delta F/F_0$ versus time t, and using the independently determined fluorescence-voltage calibration [25] (cf. Fig. 1) The temperature was 7.2°C.

that was investigated in fluorescence measurements. The transformation of the fluorescence signal into membrane voltage was performed as described before [25].

We have analysed all vesicle preparations under two different experimental conditions, immediately after the separation in the ion-exchanger column, when the vesicles still were in contact with buffer A or B before and during the measurement, and after dialysis for 24 h at 4°C in buffer containing K^+/Na^+ ratios equal to the intravesicular concentrations before chromatography. It has been checked that a period of 24 h was sufficient to restore the buffer composition and the enzymatic activity was not considerable reduced. In addition it could be excluded that, at least during the time period of the experiments (max. 5–8 h), the contact with buffer A or different concentrations of K^+, altered the transport activity of the vesicles.

All experiments have been repeated 10 times and mean and standard deviation of the determined parameters have been determined. In Fig. 5 mean values are presented. The standard deviation was in the range of 5%.

Initial rate of voltage change

For each individual vesicle the initial increase of transmembrane voltage induced by ATP can be treated as a function only of the amount of inside-out (functionally) oriented pump molecules per area of the vesicle membrane, as derived from Eqn. 1 and shown in the following: $dU_m/dt = n_p e_o v_o/AC_m$, since at low membrane potentials the leak current term, $\Lambda_m U_m$, can be neglected. The turnover rate, v_o, has been taken as constant. Therefore the initial voltage change can be used as a characteristical parameter of a sample of vesicles proportional to the average value of n_p/A. Based on previous investigations the area of the vesicle will change less than 15% from the average area of $2.5 \cdot 10^{-10}$ cm^2 in a standard preparation [28]. Assuming a specific membrane capacitance of $C_m = 1\ \mu F/cm^2$ and an initial turnover rate of $v_o = 1.8\ s^{-1}$ (at 7°C) [16], an initial slope of 1 mV/s corresponds to approximately 1 active pump per vesicle.

The initial transport activity has been investigated as function of vesicle fractionation and buffer composition. Data of four different buffer preparations and the three different vesicle fractions are compared in Fig. 5. The initial change of the transmembrane potential, dU_m/dt, is a quantity that is averaged over all vesicles in the fluorescence cuvette. As presented above the initial slope can be assumed to be the average number of pumps per vesicle. The experiments have been performed at 7.1 ± 0.2°C. One could see that the initial rate of the voltage change (or the average number of active pumps per vesicle) is different in case of unseparated preparation and fraction A and B. The differences between the four unseparated vesicle preparations had several reasons. First of all the protein had different enzymatic activities, when reconstituted in different ionic compositions, although it was taken from the same batch of Na,K-ATPase (Fig. 5 and Table I). But comparing dU_m/dt and enzymatic activity of the unseparated vesicles in Table I reveals that these differences cannot account for the total effect. The enzymatic activity of preparation 4 (0 mM K^+ + 150 mM Na^+) was reduced to 42% when compared to preparation 1 (100 mM K^+ + 10 mM Na^+). For comparison, the corresponding pumping activity was reduced to 4.3%.

TABLE I

Enzymatic activity and relative activity of vesicle fractions in different buffer compositions

Vesicles have been dialysed in buffer of 150 mM cation concentration and following composition: preparation 1 (140 mM K^+ + 10 mM Na^+); preparation 2 (50 mM K^+ + 100 mM Na^+), preparation 3 (10 mM K^+ + 140 mM Na^+) and preparation 4 (0 mM K^+ + 150 mM Na^+). The enzymatic activity, E_A, has been determined from the unseparated vesicle preparation under the conditions described in Ref. 31 and is a quantity which was controlled by the activity of the total protein concentration. The initial increase of the membrane potential, dU_m/dt, is controlled by the activity of the total protein concentration, the sidedness of the protein and the intravesicular K^+ concentration.

preparation	E_A	$(dU_m/dt)/E_A$ (mV·mg/μmol)		
	μmol/mg per h	unseparated	A	B
1	490	22.0	15.6	10.4
2	376	10.5	14.5	12.9
3	218	4.6	17.5	22.0
4	208	2.3	7.8	10.4

The pumping activity is also influenced by the intravesicular K^+ concentration. In previously published experiments vesicles have been prepared in K^+-free buffer and then redialysed to the requested K^+ concentration [40]. The initial increase of membrane potential was unchanged in a concentration range between 15 mM and 140 mM K^+. In the absence of K^+, the Na-only mode is active with a turnover rate which is approx. 15% of the physiological mode [40]. We found that in our experiments the initial slope in preparation 2 (50 mM K^+ + 100 mM Na^+) and preparation 3 (10 mM K^+ + 140 mM Na^+) are stronger reduced than the enzymatic activity. This indicated that either the turnover rate of the pump became smaller by the buffer composition or the number of active pumps in the vesicles had been modified. The reduction of the turnover rate is already reflected in the reduced enzymatic activity, since the stoichiometry between ATP hydrolysis and ion transport is constant under these experimental conditions. Therefore the differences are produced by different aggregation and/or orientation of the protein. We found that the amount of protein bound to the column increased from 48% in preparation 1 to 65% in preparation 2. Since we think that preferentially protein aggregates are bound to the column, these data indicate that the buffer composition indeed changes the conditions of reconstitution significantly. In preparation 1 the initial slope of the unseparated vesicle preparation was higher than in fraction A and B. This observation can be explained by the assumption that a very active vesicle population with a high number of inside-out-oriented pumps remained bound to the ion-exchanger matrix of the column.

To check the influence of the buffer composition on the sidedness of the pumps, we analysed the initial slope of the membrane potential of the different fractions of the vesicle preparation (Fig. 5). In preparations with high potassium during dialysis, corresponding to preparation 1, it has been found that the pump orientation was 50% inside-out and right-side out [41].

In preparation 1 (140 mM K^+ + 10 mM Na^+) the value of the initial voltage change decreased from 3 mV/s to 1.4 mV/s in the series of the unseparated, A and B vesicle samples. Vesicles from preparation 2 (50 mM K^+ + 100 mM Na^+) showed no considerable changes when fractionated, whereas in case of preparation 3 (10 mM K^+ + 140 mM Na^+) and 4 (0 mM K^+ + 150 mM Na^+) the unseparated preparation had lower pumping activity than fractions A and B (Fig. 5). On the one hand these differences show clearly that within the same preparations the chromatographic treatment separated vesicles with different number of pumps per membrane area. On the other hand it is obvious that the number of active pumps, i.e., with inside-out orientation, were different in corresponding chromatographic fractions A. No significant differences in pumping activity was found with K^+ containing vesicles eluded in high salt buffer B.

Maximum transmembrane potential

The activation of the inside-out-oriented pumps in the vesicles generates a trans-membrane potential, which reaches a maximum when the pump current is compensated by the leak current of ions through the membrane with a specific conductance Λ_m. This quasi-stationary state is characterised by the condition $dU_m/dt = 0$. This reduces Eqn. 1 to the following expression:

$$n_p e_o v = \Lambda_m A U_{max} \tag{2}$$

Where U_{max} is the maximal value of the transmembrane potential (all other quantities as given above). The turnover rate v is known to be a function of the membrane voltage and the intravesicular K^+ concentration. If we assume that the voltage dependence is not too strong, the number of pumps is approximately proportional to U_{max} in a given vesicle population. The leak conductance Λ_m depends on the amount of protein reconstituted in the vesicle independent of the orientation. Increasing amounts of protein enhance the leakage of the membranes. The dependence of U_{max} from fractionation and buffer composition were in agreement with the results obtained from the initial rate of voltage change (data not shown).

Discussion

Reconstituting Na,K-ATPase in lipid vesicles leads to inhomogeneous populations of vesicles with respect to vesicle diameter and number transport active pumps per vesicle [16,28]. These parameters may be influenced by variation of protein/lipid ratio, detergent, buffer composition and preparation method [11,17,36, 42].

The number and orientation of proteins in the vesicle membrane is crucially determined by the process of vesicle formation. A detailed study has been published in order to describe the pathway of protein-vesicle formation by the cholate-dialysis method. It was suggested that three main stages are decisive in proteoliposome formation by successive detergent removal: disc formation from mixed micelles, formation of open bilayer sheets, and closure of sheets to form vesicles [37]. The probability for the protein-lipid association depends preferentially on the concentration ratio of protein, lipid and detergent; however, the influence of other factors such as temperature, pH, salt concentration have to be taken into account too. The process of protein-lipid and/or lipid-lipid association is expected to begin when the dialysis is shifted out of equilibrium

by detergent removal from the solution in the first stage. Due to absolute quantities the fusion of the disk-like micelles with lipid-detergent micelles will be more frequent than the fusion with protein-detergent micelles. At the second stage, when large bilayer sheets are present, the probability for the protein-lipid association and/or protein incorporation will increase. As we think, the protein incorporation into the lipid-bilayer will take place mainly at this stage.

We have chosen FPLC chromatography with an anion-exchanger column to separate vesicles in defined fractions. Since it is known that proteins carry a negative charges under physiological conditions, protein-containing vesicles passing through the column become bound to the ion exchanger due to ionic interaction between proteins and the positively charged column material (Q-Sepharose). Elution with buffers of different anion concentration leads to a separation of the proteoliposomes into populations with different protein density. Since we used two salt concentrations (5 mM and 200 mM K_2SO_4), two fractions of vesicles (A and B) were obtained (Fig. 3), which contained transport active vesicles. At this state of investigation the mechanism of interaction between ion-exchanger matrix and protein is not yet clear, but it may be different for the cytoplasmic or extracellular interface of the protein.

The unseparated vesicle suspension consists mainly of unilamellar proteoliposomes and some non-vesicular material, most probably mixed micelles of protein, some lipid-aggregates and tightly bound detergent. In the fluorescence measurements the non-vesicular material contributes to the amplitude F_0 but not to the fluorescence chance induced by pumping activity. This effect causes a reduced apparent change of membrane potential and has to be taken into account in a quantitative analysis.

The vesicles have a rather narrow range of diameters [16] and the number of incorporated pumps follows a Poisson statistics according to the analysis of electron-microscopic pictures [43]. Comparing the pump activity of the unseparated vesicle suspension with fractions A and B in Fig. 5, shows that the vesicles in preparation 1 are extremely active in the unseparated solution. This activity disappeared during the chromatographic process. This finding can be explained assuming that a population of vesicles with a high number of pumps remains bound to the ion exchanger so tight that it cannot be removed by the maximum ion strength used for eluation. Vesicles that contained such a high number of pumps may have been formed by membrane fragments which were not completely dissolved during the protein solubilization. It is known that removing of Na,K-ATPase from the native membrane by detergent is accompanied by formation of compact membrane fragments containing clustered Na,K-ATPase [35]. After the centrifugation step, in which membrane particles are separated from the solubilized protein, the pellet is not very dense. Therefore, it is not unlikely that smaller fragments are taken from the pellet and included in the reconstitution process. They are thought to produce vesicles with a large number of aligned pumps.

Further evidence for the existence of these vesicles has been obtained from another set of experiments. A freshly prepared mixture of the vesicles had been centrifuged for 15 min at $100\,000 \times g$. Subsequently, we tested transport activity of the vesicles along the sedimentation gradient. The result indicated that close to the bottom of the centrifugation tubes vesicles with extremely high transport activity were collected. Normally, protein-containing vesicles are known to sediment slowly at $200\,000 \times g$ and a notable increase of the vesicle concentration at the bottom of the tube could be detected only after 6 h.

The initial voltage increase, dU_m/dt, after addition of ATP is proportional to the number of pumps with inside-out orientation. Therefore, it has been used in our further argumentation to parametrize the average pump density of the vesicles. This interpretation has been supported by the analysis of the U_{max} which are not shown in this paper, since they do not hold important additional information.

Fig. 5 and Table I demonstrate that enzymatic and pumping activity of the reconstituted Na,K-ATPase are controlled by the cationic composition of the dialysis buffer. The buffer, in which the enzymatic activity, E_A, has been determined, was the same for all vesicle preparations as in Ref. 31.

The decrease of the E_A from 490 μmol/mg per h (140 mM K^+ + 10 mM Na^+) to 208 μmol/mg per h (0 mM K^+ + 150 mM Na^+) reflects a significant loss of enzymatic activity when the K^+ contents is reduced (or Na^+ increased) during the dialysis. The effect of both ions is, that they control the distribution between the two major conformations E_1 (induced by Na^+) and E_2 (induced by K^+). Performing a reconstitution in preparation 4 (0 mM K^+ + 150 mM Na^+) with 5 mM ATP present, the pump is known to be stabilised also in conformation E_2, the enzymatic activity of the vesicle preparation is increased compared to a dialysis without ATP and comparable to a dialysis in a K^+ buffer (data not shown). These findings indicate that the pump in its E_2 conformation is more stable against denaturation during the reconstitution process.

Comparing the number of pumps ($\propto dU_m/dt$) for the vesicle fractions prepared in different buffers (Fig. 5), strikingly different patterns were found. In preparation 1 (140 mM K^+ + 10 mM Na^+) fraction A was more active than fraction B, in preparation 2 (50 mM K^+ + 100 mM Na^+) both were approximately equal. In preparation 3 (10 mM K^+ + 140 mM Na^+) and prepa-

ration 4 (0 mM K^+ + 150 mM Na^+) fraction A was less active than fraction B. Table I presents these findings as ratios of transport activity to enzymatic activity. The ratio of $(dU_m/dt)/E_A$ for the unseparated vesicles in preparation 1 to 3 decreased from 22 to 4.6. In these preparations the external buffer was in all three cases 10 mM K^+ + 140 mM Na^+, the internal buffers contained at least 10 mM K^+, therefore, the Na,K-mode of the pump was activated. The only difference in all three experiments was the ionic condition during the reconstitution. Since these numbers in Table I have been corrected for the total enzymatic activity, the number of inside-out oriented pumps must have been reduced dramatically in the unseparated preparations, when the K^+ concentration was lowered. In addition, it is obvious that the vesicles with transport-competent oriented membrane patches had disappeared in buffers with lower K^+. Possible explanations are on the one hand, that less protein is incorporated in vesicles; especially that membrane patches have been disabled to contribute. This explanation is in agreement with the observation of the amount of protein bound to the column as noted in legend of Fig. 4b. On the other hand, a non-random-oriented reconstitution (with a preference to right-side out orientation) occurred when the protein was forced into E_1 conformation during reconstitution.

Vesicles prepared in buffer containing 50 mM K^+ + 100 mM Na^+ had almost the same pumping activity in the unseparated suspension and in fraction A and B (Fig. 5). Under this condition it was not possible to separate fractions with different number of pumps. Transport competent vesicle populations with different pumping activity could be extracted from the preparation, when the reconstitution was performed with in low K^+ (< 10 mM K^+) as shown in Fig. 5. Since the unseparated solution had a significantly smaller activity than fraction A and B, a bulk of inactive material (vesicles and maybe non-vesicular aggregates) remained bound to the column. The transport activity was increased by a factor of approx. 3 (fraction A) to 5 (fraction B) compared to the unseparated vesicle preparation (Fig. 5 and Table I).

The ratio of pumping activity per enzymatic activity, $(dU_m/dt)/E_A$, is a kind of specific pumping activity. A shift of the enzyme conformation from E_2 to E_1 during dialysis (due to exchange of K^+ against Na^+ in the buffer) decreased the specific pumping activity of the vesicle preparation. Focusing on the data obtained with the Na,K-mode (preparation 1 and 3) the specific pumping activity was slightly increased in fraction A and significantly increased in fraction B when the protein was in E_1 conformation during reconstitution. The differences between Na,K-mode and Na-only mode [40] can be seen by comparison of preparation 3 and 4. The specific pumping activity for both preparations differed by a factor of approx. 2 for all three samples: unseparated vesicles, fraction A and B. This systematic difference may be accounted to the two distinct pumping modes. The distribution and orientation of the pumps in both preparations is sufficiently comparable, since the E_1 conformation is maintained equally.

Karlish and Pick [13] have shown that the enzyme orientation could not be influenced by their proteoliposome-preparation technique of freeze-thaw-sonication. At the moment we can only speculate on the reasons that produced the different orientations during reconstitution. It is known that the Na,K-ATPase molecule has an asymmetric shape. Approx. 40% of its mass protrudes into the cytoplasm and only 20% into the extracellular medium [38]. The extracellular face is decorated by sugars from the glycosylated β-subunit. In addition, it has been observed that the conformations E_1 and E_2 exhibit different proteolytic digestion pattern [5]. This can produce different topographical shapes which influence reconstitution in preformed membrane structures. As has been mentioned above the E_1 and E_2 conformations are controlled by the K^+ and Na^+ concentrations in the preparation buffer. If the major portion of protein is incorporated into the pre-formed vesicles at the last stage of the vesicles formation, then it is conceivable that differences between E_1 and E_2 conformation shift the preference to one orientation during incorporation. Another possibility is that the initial curvature of the vesicle bilayer sheet and a shape of the protein in its two conformations are two factors which could support a favoured orientation of the protein during its incorporation. A rotation of the reconstituted protein is most unlikely. Our supposition about the initial curvature of small vesicles prepared by the dialysis method is in agreement with findings in Refs. 37 and 44. The most prominent difference between the freeze-thaw-sonication method [13] and the dialysis method is the speed of the proteoliposome preparation. Formation of the proteoliposomes by sonication is very fast in a highly energized system, while formation by dialysis is performed by series of quasi equilibrium states. Therefore, the differences are not surprising and it can be expected that freeze-thaw-sonication results in a more random orientation of the protein, even if the protein is asymmetrical in his shape [13] and dialysis produces buffer controlled alignment of the protein during reconstitution.

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