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Separation and characterization of Na^+,K^+-ATPase containing vesicles

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Abstract

Na^+,K^+-ATPase was reconstituted in vesicles prepared by a dialysis method. Ion-exchange chromatography was used to obtain well characterized fractions from the inhomogeneous vesicle preparation. Lipid and protein content was determined by optical methods during the elution process. It was possible to separate fractions with distinct enzymatic and transport activities. A protocol was set up, which allowed to calculate the average number of 5-IAF labeled ion pumps per vesicle in the different fractions. The dependence of the number of protein molecules per vesicle was studied as function of the initial protein concentration added to the lipid solution before dialysis. The transport activity disappears completely at very low protein concentrations (3.3 μg protein per mg lipid). This observation is in favor of the proposal discussed in the literature, that the heterodimer $(\alpha\beta)_2$ is the transport-active form of the Na^+,K^+-ATPase. The presented method can be applied to all reconstituted vesicle preparations in which the proteins can be labeled quantitatively with a fluorescence dye.

Keywords: ATPase, Na^+/K^+-; Proteoliposomes; Ion-exchange chromatography; Fluorescence labeling; Electrogenic ion transport

1. Introduction

Phospholipid vesicles with incorporated purified membrane proteins are widely used in studies of vectorial transport processes. In reconstituted vesicles it is possible to investigate only the protein(s) of interest and to perform highly reproducible experiments. For instance, functional reconstitution of Na^+,K^+-ATPase into phospholipid vesicles was a powerful approach to study ion-transport mechanism and kinetics of conformational transitions of the enzyme [1–5]. Several methods have been used to prepare proteoliposomes with reconstituted purified Na^+,K^+-ATPase [1,6–9]. Solubilization of lipid and protein in detergents followed by removal of the detergent by dialysis proved to be a very successful method [10–13].

Experiments with proteoliposomes are often accompanied by the problem to determine quantity and orientation of the incorporated proteins. To solve the question of protein orientation in case of the Na^+,K^+-ATPase, ouabain binding to the pump before and after vesicle opening or vanadate binding to the ATP site have been investigated [14]. However, all these methods average over inhomogeneous vesicle populations. It would be more informative, if number and orientation of the proteins, which are incorporated in the vesicles, could be determined independently. Attempts to estimate the average number of the incorporated proteins have been made by different methods [3,9,14,15]. It was possible to recognize randomly distributed particles on concave and convex fracture faces of freeze-fracture electron-microscopic images. However, the absence of an adequate biochemical recognition mechanism for functional reconstituted proteins and difficulties in the statistical analysis of inhomogeneous vesicle populations [15] limit the quantification of vesicle based experiments.

A method to separate vesicle with different protein content by sucrose-gradient centrifugation of vesicle preparations has been investigated by Obermeyer [16]. However, this method turned out to be not sensitive enough to separate vesicles adequately with respect to the number of reconstituted pumps per vesicle.

A frequently noted quantity, which characterizes the protein content, is the ratio of protein to lipid content of a vesicle preparation. The protein concentration is determined normally by the Lowry method or its modifications. But this ratio is informative only under two assumptions: (i) all protein used for proteoliposome formation is incor-

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porated in the vesicles after the preparation procedure and (ii) the ratio of right-side out and reverse orientation is known. These assumptions are not fulfilled in many cases. Recently we have shown that during dialysis under certain conditions a considerable amount of protein is not incorporated in vesicles and remains in buffer forming non-vesicular protein-lipid aggregates. In addition, the orientation of the reconstituted Na^+,K^+-ATPase is dependent on the Na^+/K^+ ratio in the buffer [17]. In consequence the inaccuracy of the analysis may be high. Similar restrictions are valid for the lipid molecules. Part of lipid molecules is not assembled during vesicle formation. If a bulk lipid analysis of vesicle preparations is performed, it was found that up to 35% of the initial lipid content may be lost during dialysis.

Recently we have shown a chromatographic method which allows in principle to separate mixtures of proteoliposomes of unknown protein content into different fractions. The separation procedure is based on the interaction of negative charges of the Na^+,K^+-ATPase with quaternary amines ($-CH_2-N^{\oplus}(CH_3)_3$) of the ion exchanger material, Q-Sepharose [17]. Vesicles with different number of incorporated proteins are bound differently tight to the exchanger matrix and can be eluted by buffers with increasing salt concentrations. Aim of this work is to develop a method, which uses ion-exchange chromatography to improve the homogeneity of vesicle fractions containing defined numbers of Na^+,K^+-ATPase molecules.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids, Birmingham, AL; sodium dodecyl sulfate (SDS) from Pierce, Rockford, sodium cholate from Merck, Darmstadt. Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, special grade) were from Boehringer, Mannheim. 5-Iodoacetamidofluorescein (5-IAF) and oxonol VI (bis(3-propyl-5-oxoisoxazol-4-yl)pentamethin-oxonol) were from Molecular Probes, Junction City, OR. The dialysis tube (pore radius 2.4 nm) was purchased from Serva, Heidelberg, Q-Sepharose (a strong anion exchanger) from Pharmacia. Ion-exchange separation was performed with a FPLC System from Pharmacia, Pharmacia LKB Biotechnology, completed with a Liquid Chromatography Controller, LCC-500 PLUS, and two high precision pumps P-500. The eluate was monitored with a Fluorescence HPLC Monitor RF-535 from Schimadzu and UV-Monitor 2238 Uvicord SII from LKB. Collection of vesicle fractions was performed with a fraction collector FRAC-100 from Pharmacia. Transport experiments were carried out in a Perkin Elmer Luminometer LS-50 B.

2.2. Buffers

Buffer H: if not otherwise indicated, the buffer for vesicle experiments contained 30 mM imidazole, 1 mM EDTA, and 5 mM $MgSO_4$, the pH was adjusted to 7.2 with H_2SO_4. Buffer A: 180 mM glycerol, 1.5 mM imidazole (pH 7.2), pH adjusted with H_2SO_4. Buffer B: 200 mM K_2SO_4, 1.5 mM imidazole (pH 7.2), pH adjusted with H_2SO_4. Cholate buffer: buffer H, 1% sodium cholate, 70 mM K_2SO_4 and 5 mM Na_2SO_4.

2.3. Enzyme preparation and fluorescence labeling

Na^+,K^+-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen [11]. This method yields purified enzyme in form of membrane fragments containing about 0.6 mg phospholipid and 0.2 mg cholesterol per mg protein. The Na^+,K^+-ATPase-rich membrane fragments (about 3 mg/ml protein) were suspended in a buffer of 25 mM imidazole, 1 mM EDTA, 10 mg/ml sucrose (pH 7.5), and frozen in samples of 300 μl. This preparation could be stored for several months at -70°C without significant loss of activity. For all experiments we have chosen preparations, which were close to each other in their specific enzymatic activity. The average specific enzymatic activity was 2343 ± 140 μmol P_i per h and mg protein at 37°C.

Labeling of the enzyme with the fluorescence dye 5-IAF was performed according to Stürmer et al. [18] by incubating 200–300 μg of the enzyme for 24 h at 4°C in a solution containing 200 μM 5-IAF, 10 mM K_2SO_4 and 50 mM imidazole (pH 7.5). The labeled enzyme was separated from free dye and concentrated by centrifugation for 15 min at $100\,000 \times g$ in a Beckman airfuge at 20°C. The enzymatic activity of the protein was not altered by labeling. To measure 5-IAF fluorescence an excitation wavelength of 490 nm (slit width 10 nm) and emission wavelength of 520 nm (slit width 10 nm) was chosen.

2.4. Vesicle preparation.

The Na^+,K^+-ATPase vesicles were prepared according to a previously published method [2,9,17]. As standard preparation 2 mg/ml of the enzyme solubilized in cholate buffer were used. In order to achieve different average numbers of protein molecules incorporated in vesicles, various volumes of a lipid/detergent solution and the enzyme solubilizate were mixed, corresponding to different protein/lipid ratios: preparation 1 with about 0.7 mg protein per 7 mg lipid (1:10); preparation 2 with 0.23 mg protein per 7 mg lipid (1:30); preparation 3 with 0.07 mg protein per 7 mg lipid (1:100); and preparation 4 with 0.023 mg protein per 7 mg lipid (1:300). Between 0.8 ml and 2 ml of the combined solutions were transferred into a dialysis tubing and dialyzed for at least 72 h at 4°C against a 1000-fold volume of buffer H containing K_2SO_4 and

Na_2SO_4 in appropriate concentrations. The average enzymatic activity of the Na^+,K^+-ATPase in reconstituted vesicles was 460 ± 80 μmol P_i per h and mg protein at 37°C. Pure lipid vesicles were prepared from dioleoylphosphatidylcholine by the same procedure except addition of protein.

2.5. Analytical procedures.

The specific ATPase activity was determined by a pyruvate kinase/lactate dehydrogenase assay [19]. The enzymatic activity, E_A, of reconstituted protein was determined at 37°C according to [3]. Concentrations of protein in the enzyme solubilizate suspension, were obtained by the Lowry method [20] using bovine serum albumin as a standard. The concentration of dioleoylphosphatidylcholine in the lipid solubilizate was determined by the enzymatic phospholipid B test [21].

2.6. Ion exchange chromatography

The chromatography column (10 cm × 1 cm) was packed with 6 ml of a suspension of the anion-exchanger Q-Sepharose and equilibrated with buffer A. Typically 800–1000 μl of the vesicle solution were injected and eluted according to the following protocol: in an initial step the column was washed with 20 ml of buffer A to remove all material, which does not interact with the ion exchanger. Subsequently 20 ml aliquots with stepwise increased amounts of buffer B were flushed through the column. The increment of buffer B between two steps was 10%. The whole separation procedure was divided into 10 steps, in which the concentration of K_2SO_4 was increased stepwise by 20 mM to 200 mM. Fractions of vesicles were collected for each concentration of K_2SO_4 and analyzed separately. The elution was performed at a constant flow rate of 2 ml/min. The whole chromatographic setup was thermostatted at 4°C.

2.7. Fluorescence measurements of the initial rate of voltage change

Fluorescence experiments were carried out using a Perkin Elmer Luminometer LS-50B according to a method described elsewhere [4,17,22]. The excitation wavelength was set to 580 nm (slit width 15 nm), the emission wavelength to 660 nm (slit width 15 nm). The temperature was 14°C. The other experimental conditions of the measurement to determine the vesicles transport activity were the same as in [17]. After addition of ATP to the medium, inside-out oriented pump molecules were activated and a fluorescence increase was observed, corresponding to inside-positive potentials of up to 150–300 mV. From the initial slope of the fluorescence signal, d/dt ($\Delta F/F_0$), the initial rate of voltage change could be estimated using the independently determined fluorescence-voltage calibration with a partition coefficient for oxonol VI of 19.000 [4,17].

3. Results

When vesicles with incorporated proteins are prepared by dialysis of mixtures of solubilized protein and lipid, a considerable part of non vesicular particles (protein-lipid and lipid aggregates) can be detected in the mixture as has been shown recently [17]. These components have to be removed before the vesicles can be characterized with respect to protein and lipid content of the preparation. It is possible to separate non-vesicular particles from the vesicle suspension by using the ion-exchange chromatography. Only vesicles, with and without incorporated protein, are eluted in the absence of detergent in the elution buffer [17].

Separation from non-vesicular contaminations by column chromatography is accompanied by a 100-fold dilution of the vesicle preparations. The protein concentration in the suspension before separation was between 0.1–1 mg/ml. After separation and fractionation it was reduced to 1–10 μg/ml. Obviously it is difficult to determine the protein content with conventional methods in this low concentration range. An estimation of the lipid concentration is also of low accuracy in this range of dilution. To overcome this problem we have developed optical methods to determine protein and vesicle concentrations in diluted solutions. We detect simultaneously (i) scattering of UV light at 360 nm, which can be used to determine the lipid contents, and (ii) the fluorescence of the protein label 5-IAF, which is a direct measure of the protein concentration. In this way it was possible to characterize vesicle fractions with respect to average number of incorporated proteins per vesicle. The optical determination allowed to use the same fractions subsequently in transport studies.

3.1. Determination of the average lipid content in a suspension of proteoliposomes

The estimation of the vesicle concentration is based on the fact that light scattering by a vesicle suspension at a fixed wavelength is proportional to the vesicle concentration. This relationship can be described on the basis of Rayleigh scattering:

$$I_s = I_0 \cdot \frac{24\pi^3 v^2 c}{\lambda^4} \cdot \left(\frac{n_1^2 - n_0^2}{n_1^2 + 2n_0^2} \right) \quad (1)$$

where I_s is the intensity of the totally scattered light, I_0 is the initial light intensity, v is the vesicle volume, c is the concentration of vesicles, λ is the wavelength, n_0 and n_1 are the refraction indices of buffer and lipid vesicles, respectively. If vesicle volume, wavelength, initial light

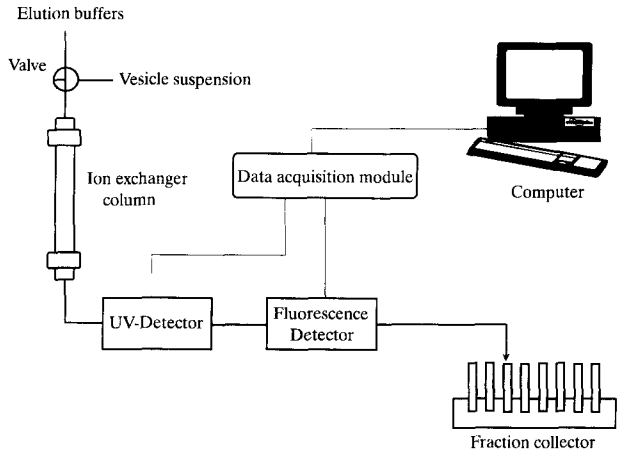

Fig. 1. Schematic presentation of the experimental setup. An aliquot of a vesicle suspension is injected into the ion-exchanger column and eluted by buffers with increasing concentrations of K_2SO_4. The eluate is analyzed by an UV detector ($\lambda = 360$ nm) and a fluorescence detector ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm, slit widths 10 nm) to obtain information on lipid and protein content. The detector outputs are collected by a data acquisition module and stored in a computer. The eluate is fractionated in samples of approx. 20 ml according to the volume of each stepwise increased salt concentration.

intensity and both refraction indexes remain constant, this relationship can be simplified to:

$$I_s = k_s c \tag{2}$$

where k_s contains all concentration independent factors of Eq. (1). Generally, the lower the wavelength the greater is parameter k_s in Eq. (2). A crucial limitation of the validity of Eq. (2) is the particle size, which causes light scattering in the solution. If the diameter of the particles is smaller than the wavelength, light reflection does not interfere with light scattering. Otherwise the dependence of the scattered light, I_s, would not be linear with respect to the vesicle concentration, c. The average vesicle diameter of 96 nm [2] is much smaller than the used wavelength of 360 nm. At this wavelength absorption of the protein has not to be taken into account, it has its maximum at 270 nm.

The principle of the experimental setup is shown on the Fig. 1. 800–1000 μl of a vesicle containing solution were injected into the ion-exchanger column and eluted by buffers with increasing concentrations of K_2SO_4. The eluate from the chromatographic column was passed through the flow-through cell of the UV detector and with a delay of 400 μl through the flow-through cell of the fluorescence detector. Fractionated vesicles were sampled in a fraction collector. The readout of both detectors was sampled in parallel by a data acquisition board and stored in a computer for further evaluation.

Calculation of the vesicle concentrations were performed by measuring the integral optical density D_{int}, which is related to the intensity of the scattered light as shown in Appendix A. We measured D_{int} with vesicle solutions of known lipid concentrations. From a well characterized preparation of lipid vesicles without protein, various dilutions were made and samples of 800 μl with lipid contents between 140 μg and 1.8 mg were injected and passed through the column and detectors. The temperature was 4°C. Vesicles without protein were not bound to the ion-exchanger matrix of the column [17]. A linear dependence between amount of lipid, n_{lipid}, and integral optical density, D_{int}, according to Eq. (A7) was observed in the whole concentration range. The slope, k_D, was determined to be (0.70 ± 0.02) ng^{-1}.

From the lipid concentration we can estimate the number of vesicles in the solution using the following assumptions. According to a radius of the head group of 4.72 Å [23] the space occupied by a lipid molecule can be assumed to be 70 Å^2. Protein containing vesicle have an external diameter of (96 ± 10) nm [2]. The molar weight of the lipid is 768.15 g/mol. If the lipid layer is supposed to be packed with the highest possible density of the lipid head groups, a vesicle contains approx. 70000 lipid molecules. This corresponds to approx. $1.1 \cdot 10^{10}$ vesicles per 1 μg lipid in the solution.

3.2. Determination of the average protein content in a suspension of proteoliposomes

The high dilution of proteoliposomes in the suspension after chromatographic separation made it necessary to develop an appropriate method to determine the protein concentration. Labeling the protein with the fluorescence dye 5-IAF allows to detect concentrations in the range of 10^{-2}–10^{-3} mg/ml. The dependence of fluorescence intensity on protein concentration was detected under stationary conditions, in which different solutions with known concentration of labeled and solubilized Na^+,K^+-ATPase were measured in a cuvette at 4°C. The inset in Fig. 2 shows a linear relation between fluorescence and protein concentration in the whole range. Experiments were performed, in which defined portions of solubilized protein (between 20 ng and 5 μg) were passed through the fluorescence detector with a constant flow rate of 2 ml/min. In these experiments the protein was not passed through the column. In Fig. 2 the integrated fluorescence intensity, F_{int}, is plotted against the amount of injected protein. The linear dependence proved that F_{int} can be used to determine the protein content, n_{prot}.

$$F_{int} = k_f n_{prot} \tag{3}$$

From data in Fig. 2 the value of k_f was calculated to be (0.54 ± 0.01) μg^{-1} in our setup.

Control experiments with pure lipid vesicles showed that the fluorescence detector measured also some intensity in the absence of 5-IAF, caused by light scattering. This monochromator-induced artifact has to be taken into account by splitting the measured apparent fluorescence in-

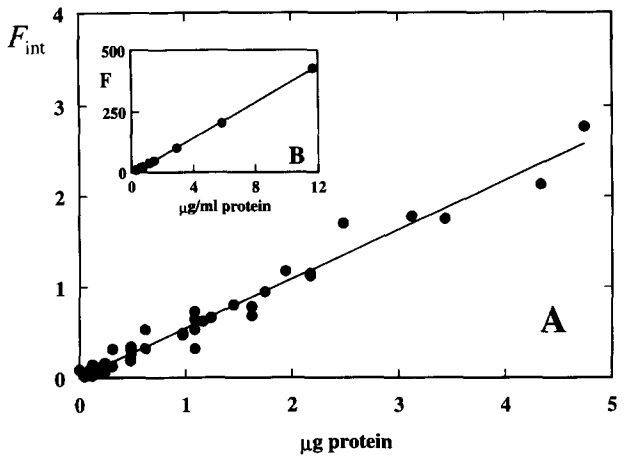

Fig. 2. Integral fluorescence intensity, F_{int}, as function of the amount of 5-IAF labeled Na^+,K^+-ATPase. The labeled protein was solubilized in buffer containing sodium cholate. Samples with a protein contents between 20 ng an 5 μg were passed through the fluorescence cell and the fluorescence intensity was recorded and integrated. A linear correlation between integrated fluorescence intensity and amount of protein according to Eq. (3) was found in the whole concentration range. The monochromators were set to $\lambda_{ex} = 490$ nm (slit width 10 nm), $\lambda_{em} = 520$ nm (slit width 10 nm). The temperature was 4°C. Inset: The same relation was found in measurements, which were performed in a standard fluorescence spectrophotometer with protein samples in the concentration range of 0.3–12 μg/ml. Even the lowest protein concentration produced a significant fluorescence intensity above background.

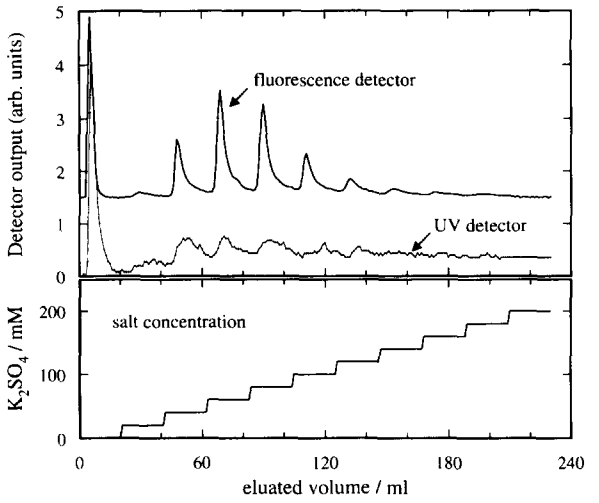

Fig. 3. Typical elution protocol of an inhomogeneous vesicle preparation. In the initial step the column was washed with 20 ml of buffer A to remove all material, which does not interact with the ion exchanger. The high elution peak was not caused by overloading the column. Subsequently 22.5 ml aliquots with stepwise increased amounts of buffer B were flushed through the column. The lower panel shows the corresponding K_2SO_4 concentrations in the elution buffer. Above 100 mM K_2SO_4 ($V_{elution} > 120$ ml) the UV signal, which detected the vesicle content, could be analyzed only with large errors. The temperature was 4°C.

tensity, F_Σ, into dye fluorescence, F_d, and the light-scattering induced component, I_s:

$$F_\Sigma = F_d + I_s \quad (4)$$

On the basis of this relation the integral dye fluorescence, $F_{d,int}$, could be expressed by:

$$F_{d,int} = F_{\Sigma,int} - D_{int} k_r \quad (5)$$

where $F_{d,int}$ is the integral dye fluorescence, $F_{\Sigma,int}$ is the measured integral fluorescence intensity, k_r, is the light scattering correction factor (for details see Appendix B).

When the corresponding integrals of the optical density, D_{int}, and scattered light, $I_{s,int}$, in the fluorescence detector are plotted against each other, a straight line in agreement with Eq. (B2) was found in the whole range of D_{int}. A regression line through the data was used to determine k_r, which is 1.16 ± 0.03 for our setup. The experimentally determined contribution of scattered light to the fluorescence signal, $D_{int}k_r$, was in the range of 3 to 10% of the total signal depending on the concentration of 5-IAF-labeled protein in the vesicle containing fraction.

In Fig. 3 a typical elution protocol of a vesicle preparation is presented. An aliquot of 800 μl was injected into the column and eluted under the conditions described above. The volume of a particular concentration step of K_2SO_4 was 22.5 ml. This volume allowed to collect quantitatively vesicles, which were eluted by each different ion concentration. The optical density and the fluorescence intensity were integrated over the total elution volume of each concentration step. For each fraction the lipid content, n_{lipid}, was calculated according to Eq. (A7) and the protein, n_{prot}, according to Eq. (3). The numerical analysis of a vesicle preparation 2 is presented in Fig. 4A. Additional tests with lower concentrations of vesicles made sure that the column was not overloaded. In the first fraction, eluded by buffer A, protein-containing and protein-free vesicles were obtained [17]. In the second fraction with 20 mM K_2SO_4 almost no protein could be detected, for salt

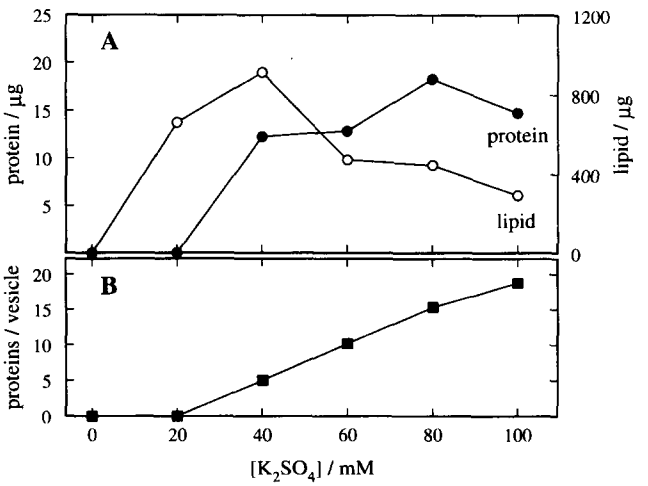

Fig. 4. Estimation of the protein to lipid ratio. (A) Optical data of an elution protocol (as shown in Fig. 3) were integrated with respect to each concentration of K_2SO_4, and D_{int} and F_{int} were used to determine the amount of lipid and protein in each sample according to Eqs. (3), (5) and (A7). (B) As described in the text, the results of panel (A) may be used to calculate the average number of pumps per vesicle in each sample.

concentrations above 100 mM detection of eluted lipid approached zero (Fig. 3).

To calculate the number of the protein molecules per vesicle the following assumptions were made: The number of vesicles per μg of lipid was estimated as described above. The molar weight of an protomer of the Na^+,K^+-ATPase is 147 kDa. This weight was used, although it is not yet clear if this is the smallest active unit of the ion pump. If the dimer $(\alpha\beta)_2$ forms the pump, the calculated number has to be divided by 2. On the basis of the numbers given in Fig. 4A, the protein molecules per vesicle were determined and plotted in Fig. 4B. It should be noted that the protein per vesicle ratio was computed only for vesicle fractions, which produced significant signals in both detectors. The smallest detectable amount of the lipid in a fraction was 140 μg. Due to this restriction usually vesicle fractions eluted in the concentration range of K_2SO_4 above 100 mM were not analyzed.

3.3. Stability of the 5-IAF binding

The quantitative analysis of the protein content is based on the intensity of the 5-IAF fluorescence. This label is bound to the protein with a 1:1 stoichiometry to an $\alpha\beta$ monomer. In a series of experiments the stability of 5-IAF binding was tested. As mentioned above, the vesicles were prepared by a 72 h dialysis of a mixture of solubilized lipid and labeled protein. To check the influence of the age of a preparation on the amount of bound 5-IAF label, proteoliposomes were prepared with a protein content 0.23 mg protein per 7 mg lipid (preparation 2). Aliquots were separated immediately after dialysis as well as 2, 5, and 8 days later. The protein content was determined and is presented in Fig. 5 for all four series. The small variations within the same eluted fractions indicate strongly that there is no age dependent loss of the 5-IAF label. When free dye

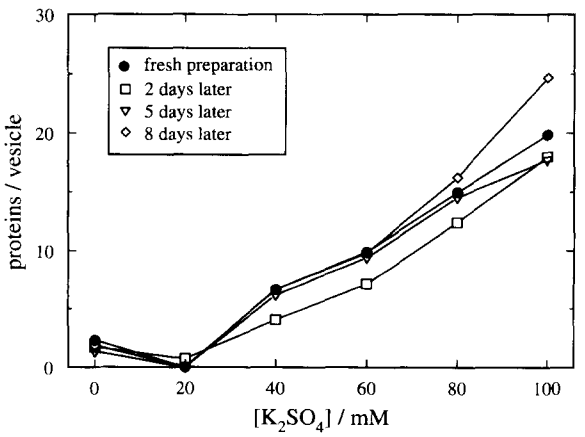

Fig. 5. Stability of the 5-IAF binding. A vesicle preparation of type 2 was fractionated at different times after the dialysis (0, 2, 5, 8 days). From the UV and fluorescence signals the number of pumps per vesicle was determined. No significant deviations could be detected during this period within the samples eluted by the same K_2SO_4 concentration.

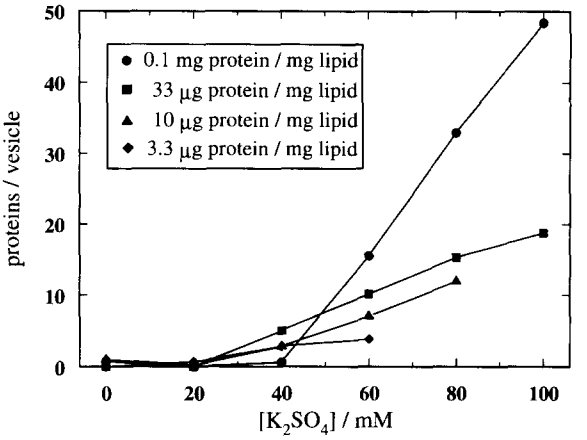

Fig. 6. Average protein content of vesicle fractions as function of the K_2SO_4 concentration in the elution buffer for vesicle preparations with different initial protein concentrations. The chromatography was performed at 4°C.

was injected, it remained bound to the column and could be removed only by buffers which contained detergent.

3.4. Separation of the vesicles with various protein content and their characterization

The chromatographic separation of a vesicle preparation as shown in Fig. 4 was repeated with preparations of various initial protein content (preparations 1, 2, 3 and 4, see Section 2.4). In Fig. 6 for all four preparations the number of proteins per vesicle are presented according to Fig. 4B. It has to be mentioned that not all separated fractions could be evaluated with respect to their protein and lipid concentration. In preparations 3 and 4 the concentration of vesicles was so small in fractions above 80 or 60 mM K_2SO_4, respectively, that the corresponding optical signals could not be evaluated.

A common feature of all separations was that both, pure lipid vesicles and protein containing vesicles, were eluted with buffer A and at a salt concentration of 20 mM K_2SO_4 almost no protein containing vesicles could be detected. Preparations 2, 3 and 4 exhibited comparable elution pattern, preparation 1 with the highest protein/lipid ratio deviated significantly at salt concentrations above 60 mM K_2SO_4 (Fig. 6).

3.5. Initial rate of voltage change

Inside-out oriented Na^+,K^+-ATPase molecules can be activated by ATP to generate a transmembrane potential by ion pumping. For each individual vesicle the initial increase of transmembrane voltage depends only on the number of inside-out oriented pumps in the vesicle membrane and their turnover rate [17]. The membrane-potential sensitive fluorescence dye oxonol VI allowed to determine the pump-induced change of membrane potential [4,5,17]. In all presented experiments the conditions of the oxonol

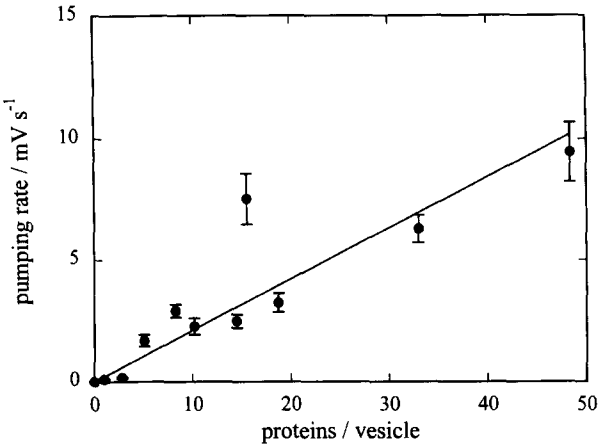

Fig. 7. Pumping rate of the reconstituted Na^+,K^+-ATPase as function of the corresponding average number of protein molecules per vesicle determined from single fractions of different vesicle preparations. The error bars represent the scattering of data between different repetitions of the experiments.

experiments were the same. All 4 vesicle preparations were dialyzed in the same buffer and protein was taken from the same batch. Therefore it was justified to assume that the turnover rate of a single pump did not vary in all analyzed experiments and the overall pumping rate of a vesicle depended only on the number of pump molecules per area of vesicle membrane. Accordingly the initial rate of the transmembrane voltage change should increase proportionally to the number of inside-out reconstituted proteins per vesicle. In Fig. 7 we have compiled the results of the oxonol experiments from all vesicle preparations and plotted the pumping rate of each vesicle fraction against the corresponding calculated number of pumps. Each point represents the mean value of at least 6 experiments. Fig. 7 shows a linear dependence of the pumping rate on the number of incorporated pumps.

4. Discussion

4.1. Separation and characterization of the vesicle fractions

Since the first study of protein reconstitution in vesicles has been published in 1965 [24], a variety of techniques were developed and tested to extend this method for different purposes and applications [10,13,25–28]. Reconstitution of membrane proteins in the lipid vesicles is a powerful method to construct a model system with relative constant and reproducible properties. However, it was not possible so far to design a method, which produces proteoliposomes of defined size and protein content and orientation. These informations are essential for a quantitative analysis of the transport function and characterization of reconstituted proteins. Therefore we studied a method, which allowed to separate different fractions from a vesicle preparation, which has been used widely for reconstitution of Na^+,K^+-ATPase [2,6,13,17].

Ion-exchange chromatography with Q-Sepharose was used to fractionate vesicle suspensions into several samples with respect to average protein content (Fig. 3 and 4). Due to the high dilution of the vesicles after elution from the column, it was impossible to use the Lowry method to determine the protein concentration in the eluate. For the Na^+,K^+-ATPase the lowest concentration level which could be analyzed by the standard Lowry method is in the range of 0.1–1.0 mg/ml. After the chromatographic separation the protein concentration decreased to 0.1 μg per fraction of 20 ml. The determination of lipid concentration in the separated vesicle fractions was accompanied by comparable difficulties. To overcome the problem of high dilution, we developed optical methods to detect simultaneously the concentration of lipid (vesicles) and protein with an improved sensitivity. We made use from the fact that Na^+,K^+-ATPase can be labeled covalently by the fluorescence dye 5-IAF without changing its enzymatic properties [18,29]. The lowest concentration of protein, which could be discriminated definitely from background with a fluorescence detector, was about 0.2 μg/ml (Fig. 2). Integrating the fluorescence signal over a whole fraction of 20 ml, minimum amounts of protein in the range of 0.1 μg were traced. This number has to be compared with the standard Lowry method, in which a minimum detectable concentration is typically 2 μg Na^+,K^+-ATPase in 50 μl. The lipid content of the chromatographically obtained fractions was determined by the amount of UV light scattered by vesicles, which have a rather uniform size distribution of 96 ± 10 nm [2,15]. The lowest amount of vesicles, which was accepted as significant in a fraction of separated vesicles, corresponded to 140 μg of lipid. These experimental limitations allowed to characterize only five fractions, although the salt gradient was increased in 10 steps (Fig. 3). Generally the detection of lipid (or vesicles) was limiting. In principle there is a possibility to improve the detector sensitivity and to diminish the detectable amount of vesicles. If the wavelength of the scattered UV light would be reduced (from 360 nm as used in this work) according to Eq. (1) the intensity of scattered light would increase proportional to $1/\lambda^4$. Selecting a wavelength of 303 nm instead of 360 nm will double the optical density detected by the UV detector (Eq. A4).

Since the protein content of vesicle containing solutions is determined by the fluorescence label 5-IAF, it was crucial to make sure that the label remained bound to the pump during the period of experiments. Therefore aliquots of the same batch were analyzed between immediately after dialysis and 8 days later. In all four series of experiments during that period the optically determined numbers of proteins per vesicles did not change significantly in the different fractions (Fig. 5) and the ion-pumping rate of the inside-out oriented proteins, which was measured by ox-

onol experiments corresponded similarly to the number of proteins per vesicle (data not shown).

4.2. Influence of the initial protein concentration

Vesicles were prepared with different initial concentrations of protein between 3.3 μg per mg lipid (preparation 4) and 100 μg per mg lipid (preparation 1). If all protein molecules behave statistically independent, reconstitution of the proteins into vesicles can be expected to follow a Poisson distribution [15]. Assuming that the orientation of pumps (inside-out or right-side out) is independent of protein concentration and that no aggregation of protein in the vesicle membrane does occur, it should be expected that in all preparations for a given ionic strength a fraction of vesicles is eluted, which has the same number of pumps per vesicle. The corresponding results of preparations 1-4 are presented in Fig. 6. All preparations were made at the same time from the same batch of protein. While in preparations 1-3, with lower protein concentrations (3.3-33 μg protein per mg lipid), the deviations were not very significant, preparation 1 with 0.1 mg protein per mg lipid showed a remarkable different behavior. We observed that at 20 mM K_2SO_4 no protein containing vesicles were eluted from the column in all four preparations, whereas in buffer without K_2SO_4 considerable amounts of pure lipid vesicles and protein containing vesicles could be washed off the column (Fig. 4). Preparations 1 to 3 fulfill in the concentration range above 20 mM K_2SO_4 approximately a linear relation between the number of proteins per vesicle and the salt concentration in the buffer. The slope of the curves can be calculated formally to be 0.2–0.25 proteins per mM K_2SO_4 in the elution buffer. The corresponding slope for preparation 4 would be 0.8 proteins per mM K_2SO_4 in the concentration range above 40 mM.

The most reasonable explanation of the deviations, which were found in preparations of type 1, was that the assumption of independently distributed pumps in the membrane may be no longer correct when the protein concentration becomes as high as 0.1 mg protein per mg lipid. Aggregation of Na^+,K^+-ATPase molecules in similar preparations was discussed in recent publications [16,17]. Obermeyer estimated from results of density-gradient centrifugation that the pump is reconstituted in oligomers of a molecular mass close to 1000 kDa [16]. If the Na^+,K^+-ATPase is able to aggregate during the reconstitution process in the case of a high enough number of proteins per vesicle, the density of charges on the vesicle surface may become higher than the density of counter charges on the ion-exchanger matrix. On the basis of a ratio of 0.1 mg protein per mg lipid about 25 inside-out oriented $\alpha\beta$ units of the Na^+,K^+-ATPase (with 147 kDa) may be expected as an average in a vesicle of preparation 1 [2]. Even if the pumps are organized as heterodimers $(\alpha\beta)_2$ the probability of aggregation is rather high. As a consequence, a given salt concentration will exchange vesicles with a higher number of pumps compared to vesicles without oligomers of the protein. In preparations of type 2 to 4 the average numbers of pumps is reduced by a factor of 3, 10 and 30, respectively.

To check the relation between the calculated number of pumps per vesicle in separated fractions and the corresponding transport activity, the ion-pump activity was determined by fluorescence experiments, in which the pump-generated membrane potential can be measured by oxonol VI [4,5,13,17]. The pumping rate was calculated and plotted against the number of pumps in Fig. 7. A linear relation was observed as indicated by the regression line, which is drawn in Fig. 7. The slope corresponds to 0.2 mV/s per $\alpha\beta$ unit in a vesicle (without taking the orientation of the pumps into account).

It is noteworthy to mention that all preparations with less than 5 $\alpha\beta$ units per vesicle did not showed significant transport activity, i.e., the pumping rate was smaller than 0.2 mV/s and was therefore comparable to unspecific drift of the fluorescence intensity. This could be observed with all fractions of preparations of type 4 (3.3 μg protein/mg lipid). These fractions contained 4 or less $\alpha\beta$ units in a vesicle. Two or more active pumps produce a pumping rate of at least 0.4 mV/s. This can be detected with the mentioned sensitivity of the oxonol VI method. If the Na^+,K^+-ATPase works as $\alpha\beta$ monomer, due to statistical reasons 50% or more of the vesicles in fraction with 3 or 4 $\alpha\beta$ should have reconstituted at least two pumps inside out and their activity would be detectable. If it is assumed that $(\alpha\beta)_2$ is the minimal transport active form of the Na^+,K^+-ATPase, the probability is significantly reduced to find a functional inside-out oriented pump in vesicles with protein density as low as in preparation 4. Therefore the presented findings can be interpreted as being in favor of the model of a heterodimer, $(\alpha\beta)_2$, as the minimal requirement for a transport active Na^+,K^+-ATPase.

The presented method allows the fractionation of heterogeneous vesicle preparations as they are obtained by different reconstitution techniques. The use of fluorescence-labeled protein allows the quantification of number of proteins per vesicle by optical detection in a range beyond the sensitivity of standard techniques as the Lowry method. The disadvantage of the dilution of the resulting fractions can be overcome by concentration procedures. However, for many transport studies, e.g., with potential sensitive fluorescence dyes, the vesicle concentration is still sufficiently high.

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Appendix

Appendix A: Integral optical density

The UV detector measures light scattering as a decrease of the incident light according to the law of Lambert and Beer

$$I = I_0 e^{-\tau l} \tag{A1}$$

where I is the detected light intensity, I_0 the incident intensity, l is the optical length of the cuvette and τ is the turbidity of the solution which is defined by

$$\tau = \frac{24\pi^3 v^2 c}{\lambda^4} \cdot \left(\frac{n_1^2 - n_2^2}{n_1^2 + 2n_2^2} \right) \tag{A2}$$

The parameters are the same as given in Eq. (1). The optical density of the solution, D, as measured by the UV detector, is defined by

$$D = \ln \frac{I_0}{I} \tag{A3}$$

From Eqs. (A1) to (A3) the optical density, D, can be written as

$$D = \tau l = \frac{24\pi^3 v^2 c}{\lambda^4} \cdot \left(\frac{n_1^2 - n_2^2}{n_1^2 + 2n_2^2} \right) \cdot l \tag{A4}$$

If volume of the vesicle, wavelength, refraction indices, and the length of the optical way of the cuvette remain constant, Eq. (A4) can be simplified to

$$D = k_D c \tag{A5}$$

Since the amount of lipid, which passes through the detector, varies with time during the elution procedure, for each particular volume, dv, the optical density has to be detected and the lipid content has to be determined by $\Delta v \cdot c$ and c can be calculated from D according to Eq. (A5). The integral optical density, D_{int}, can be determined by integration of all volumes dv during the elution of volume $\Delta V = v_1 - v_0$:

$$D_{int} = \int_{v_0}^{v_1} D \cdot dv \tag{A6}$$

The amount of lipid, n_{lipid}, is linked to D_{int} by

$$D_{int} = k_D n_{lipid} \tag{A7}$$

Appendix B: Light scattering correction factor

According to Eqs. (2) and (A5) the ratio of scattered light, I_s, in the fluorescence detector and the optical density, D, determined in the UV detector have to be independent from the vesicle concentration.

$$\frac{I_s}{D} = \frac{k_s c}{k_D c} \equiv k_r \tag{B1}$$

The same relation is valid for the integral optical density, D_{int}, (Eq. A6) and the integral scattered light, $I_{s,int}$, which can be introduced similarly, so that

$$\frac{I_{s,int}}{D_{int}} = k_r \tag{B2}$$

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