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## **(Na<sup>+</sup> + K<sup>+</sup>)-ATPase in artificial lipid vesicles: influence of lipid structure on pumping rate**

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**(Na<sup>+</sup> + K<sup>+</sup>)-ATPase from kidney outer medulla was incorporated into tightly-sealed, single-shelled lipid vesicles by a detergent-dialysis procedure. The rate of ATP-driven potassium extrusion from vesicles formed from different phosphatidylcholines (PC) was measured optically, using a voltage-sensitive dye in the presence of valinomycin. High transport rates were observed for di(18:1)PC, di(20:1)PC and di(22:1)PC, whereas vesicles formed from di(14:1)PC and di(16:1)PC were virtually inactive. The variation of pumping activity with lipid structure mainly results from differences in the amount of enzyme incorporated with the correct orientation into the vesicle membrane, and to a lesser extent from lipid-dependent variations of the intrinsic turnover rate of the enzyme. The activation energy of ion transport decreases in the order di(16:1)PC, di(18:1)PC, di(20:1)PC ≈ di(22:1)PC.**

### **Introduction**

The (Na<sup>+</sup> + K<sup>+</sup>)-pump, like other transport ATPases of biological membranes, requires lipid for its function [1]. Removal of lipid by detergent extraction or phospholipase treatment leads to a loss of activity; relipidation partially or fully restores function [2–12] (see Ref. 1 for a survey of the earlier literature). Experiments in which the native lipid is removed and replaced by chemically defined lipids offer the possibility of studying the influence of lipid structure on the activity of the (Na<sup>+</sup> + K<sup>+</sup>)-ATPase [1–5]. Functional reconstitution requires that the pump protein is incorporated into the membrane of tightly sealed lipid vesicles so that ATP-driven ion translocation across the vesicle membrane can be observed. However, in most reconstitution experiments with lipids of known structures which have been carried out in

the past [2–5] only the enzyme activity of the (Na<sup>+</sup> + K<sup>+</sup>)-ATPase could be measured after association of the protein with the lipid. It is not clear whether in these experiments the pump molecule is incorporated in such a way that it spans the bilayer and whether in the enzymatic test ATP-hydrolysis is coupled to ion translocation.

In the following, we describe reconstitution experiments with chemically defined phosphatidylcholines using a detergent dialysis method, which yields tightly sealed, single-shelled vesicles [13–15]. With these vesicles ATP-driven potassium extrusion has been studied by an optical technique using a potential-sensitive dye [12]. In the presence of valinomycin the voltage across the vesicle membrane approximates the Nernst potential for potassium. Measuring the transmembrane voltage, the time-course of K<sup>+</sup> concentration in the vesicle interior can be followed. In this way rates and

activation energies of pump-mediated ion transport have been determined in vesicles made from phosphatidylcholines with different hydrocarbon chains.

### Materials and Methods

**Materials.** L- $\alpha$ -Phosphatidylcholines (PC) with the following mono- and diunsaturated fatty-acid chains were obtained from Avanti Polar Lipids Inc., Birmingham, AL: dimyristoleoylPC (di(14:1)PC), dipalmitoleoylPC (di(16:1)PC), dioleoylPC (di(18:1)PC), dilinolenoylPC (di(18:2)PC), dilinolenoylPC (di(18:3)PC), dieicosenoylPC (di(20:1)PC) and dierucoylPC (di(22:1)PC). Egg PC was prepared by K. Janko according to the method of Singleton et al. [26]. Sodium dodecyl-sulfate (SDS) was obtained from Pierce Chemical Company, Rockford, IL, and sodium cholate from Merck, Darmstadt. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, Sonderqualität) were from Boehringer, Mannheim. 1,3,3,1',3',3'-hexamethyl-indodicarbocyanine (NK 529) was purchased from Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan.  $^{137}\text{CsCl}$  was from New England Nuclear. All other reagents were obtained from Merck (analytical grade). Dialysis tubing was purchased from Serva, Heidelberg.

**Buffer H.** If not otherwise indicated, the buffer for the vesicle experiments (buffer H) contained 30 mM imidazole, 1 mM L-cysteine, 1 mM EDTA and 5 mM  $\text{MgSO}_4$ ; the pH was adjusted to 7.2 with  $\text{H}_2\text{SO}_4$ .

**Enzyme preparation.** ( $\text{Na}^+ + \text{K}^+$ )-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen [16]. This method yields purified enzyme in the form of membrane fragments containing about 0.6 mg phospholipid and 0.2 mg cholesterol per mg protein [16,17]. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [18] and the protein concentration was determined by the Lowry method [19], using bovine serum albumin as standard. For all preparations the specific activity was in the range 1500–2200  $\mu\text{mol P}_i/\text{h}$  per mg protein at 37°C.

**( $\text{Na}^+ + \text{K}^+$ )-ATPase vesicles.** The purified ( $\text{Na}^+ + \text{K}^+$ )-ATPase was solubilized in sodium cholate as described previously [12,20]. 2 mg of the

enzyme were suspended in 1 ml 'cholate buffer' consisting of buffer H with 23 mM sodium cholate and 70 mM  $\text{K}_2\text{SO}_4$  plus 5 mM  $\text{Na}_2\text{SO}_4$  ( $\text{SO}_4^{2-}$  was used as anion instead of  $\text{Cl}^-$  in order to minimize passive anion permeation). After agitation of the suspension for 30 s with a high-speed Vortex mixer, undissolved material was separated from the solubilise by centrifugation for 15 min at  $100\,000 \times g$  (Beckman, Airfuge).

A solution of the appropriate lipid in chloroform was evaporated under a stream of  $\text{N}_2$  in a round-bottom flask to yield a thin film on the glass wall. The lipid was dissolved in a 1% (w/v) cholate solution in methanol to yield a concentration of 20 mg lipid/ml. After vacuum evaporation of the methanol, buffer H was added to a final concentration of 1% cholate. Equal volumes of the resulting solution and the enzyme solubilise were mixed, corresponding to a protein:lipid ratio of about 60  $\mu\text{g}$  protein per mg lipid. 200  $\mu\text{l}$  of the combined solubilise were transferred to 7 mm dialysis tubing and dialysed for 60 h at 4°C against 200 ml buffer H containing 70 mM  $\text{K}_2\text{SO}_4$  and 5 mM  $\text{Na}_2\text{SO}_4$ .

The size distribution of the vesicles was studied by dynamic light scattering [21,24] using a scattering photometer equipped with an argon ion laser (Spectra Physics; wavelength 514.5 nm). From the correlation time of the intensity of scattered light (scattering angle 90°), the average translational diffusion coefficient,  $D$ , of the vesicles was determined [21]. Assuming spherical shape, the average vesicle radius,  $r$ , was calculated using the Stokes-Einstein relation  $D = kT/6\pi\eta r$  ( $k$ , Boltzmann's constant;  $T$ , absolute temperature;  $\eta$ , viscosity of the solution). From the deviation of the correlation function from a purely exponential time dependence, the variance,  $\sigma$ , of the distribution of vesicle radii was estimated [24]. Within the limits of experimental error,  $\sigma$  was almost identical for the different vesicle preparations. A value of  $\sigma = 10$  nm was used throughout.

The lipid content of the vesicle suspension after dialysis was determined by the phosphorous method [36] or by high-pressure liquid chromatography on a LiChrosorb Si 60 column (Knauer, Bad Homburg, F.R.G.) with methanol/water as solvent. The loss of lipid during dialysis was found to be less than 10%.

For some of the preparations the size of the entrapped aqueous space of the vesicles was determined by adding  $^{137}\text{Cs}^+$  to the vesicle suspension. After variable time periods, the aqueous medium was separated from the vesicles by Sephadex G-50 gel chromatography and the radioactivity of the vesicles was counted [22]. A period of 24 h was found to be sufficient for complete equilibration of  $^{137}\text{Cs}^+$  between vesicle interior and medium.

**Fluorescence measurement.** Fluorescence measurements were carried out with a Perkin-Elmer 650-40 fluorescence spectrophotometer, as described previously [12]. The thermostated cell holder was equipped with a magnetic stirrer. The cell was filled with 1 ml of buffer H containing 5 mM  $\text{K}_2\text{SO}_4$ , 70 mM  $\text{Na}_2\text{SO}_4$  and  $2.3 \mu\text{M}$  1,3,3,1',3',3'-hexamethylindodicarbocyanine. The dye was added from a 0.1% (w/v) stock solution in 1:9 (v/v) ethanol:water.  $5 \mu\text{l}$  of the vesicle suspension containing approx. 10 mg lipid/ml was added to the solution in the cell. After the fluorescence signal had reached a steady value, 20 nM valinomycin was added from a concentrated stock solution in ethanol. Addition of the same amount of ethanol had virtually no effect on the fluorescence. The solution in the cell was continuously stirred. The time resolution of the measurement was limited by the mixing time after reagent addition to the cell and was about 3 s.

**Calibration of fluorescence signals.** The relative fluorescence signal,  $\Delta F/F_0$ , was calibrated as a function of membrane voltage,  $U$ , by creating a gradient of potassium concentration across the vesicle membrane in the presence of the potassium-ionophore valinomycin [12,23]. Under this condition the membrane voltage is approximately equal to the Nernst potential for potassium. The fluorescence signal was shown previously to be a unique function of the Nernst potential, irrespective of the absolute magnitude of potassium concentration [12]. As seen in Fig. 1, the data points in the plot of  $\Delta F/F_0$  vs.  $U$  for all lipids (except for di(22:1)PC) are close to the line for di(18:1)PC which has been taken from a preceding paper [12]. The analysis of the flux experiments was based on the calibration curve for di(18:1)PC with the slope of the curve corrected for each lipid according to Fig. 1.

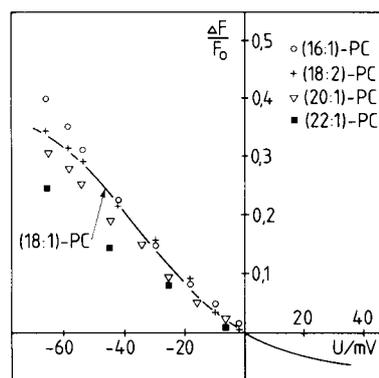


Fig. 1. Calibration of the fluorescence signals as a function of membrane voltage,  $U$ .  $F_0$  is the fluorescence signal prior to the addition of valinomycin and  $\Delta F$  is the fluorescence increment in the presence of valinomycin at a given ratio of the external and internal potassium concentration. The membrane voltage,  $U$ , was calculated using the Nernst relation. Each data point is the average of 4–5 measurements with different vesicle suspensions. The line describing the results for di(18:1)PC has been taken from Ref. 12. The temperature was  $25^\circ\text{C}$  for di(22:1)PC and  $16^\circ\text{C}$  for the other lipids.

**Data analysis.** From the time-course of membrane voltage,  $U$ , after ATP addition to the medium at time  $t = 0$ , the potassium concentration  $c_{\text{K}}^i(t)$  in the vesicle interior is obtainable using the Nernst equation. The time derivative of  $c_{\text{K}}^i$  is related to the turnover rate,  $v$ , of the pump and the average number,  $n_p$ , of pump molecules per vesicle (ATP-binding site facing outward);

$$3n_p v = -V \frac{dc_{\text{K}}^i}{dt} \quad (1)$$

$V$  is the average volume of the entrapped aqueous phase of a vesicle. The factor 3 in Eqn. 1 results from the fact that in the presence of valinomycin the overall exchange of  $\text{Na}^+$  and  $\text{K}^+$  is electroneutral; this means that three potassium ions leave the vesicle per pump cycle (assuming a  $\text{Na}^+/\text{ATP}$  stoichiometry of 3:1). Using Eqn. 1 the initial turnover rate,  $v_0$ , may be obtained, in principle, from the value of  $dc_{\text{K}}^i/dt$  at zero time. As discussed previously [12,27], this method is unsatisfactory, since the vesicle population is heterogeneous with respect to size and number of pump molecules per vesicle. Vesicles with a large  $n_p/V$  ratio lose their internal  $\text{K}^+$  at a high rate which makes the determination of  $v_0$  inaccurate at the

limited time resolution of the fluorescence experiment. For a more quantitative analysis the heterogeneity of the vesicle population has to be taken into account explicitly. As in a previous paper [12], the analysis has been based on the assumption of a normal distribution of vesicle radii and of a Poisson distribution of the number of pump molecules per vesicle. By fitting the theoretical equation given in Ref. 12 to the experimentally observed time-course of the fluorescence signal (by the method described in the Appendix of Ref. 12), the initial pump rate,  $v_0$ , and the density,  $\chi$ , of molecules per unit membrane area can be determined. The average (outer) vesicle radius,  $r = d/2$ , which enters into the analysis was taken from Table I, and the entrapped volume,  $V$ , was calculated according to  $V = (4\pi/3)r_i^3$ , where  $r_i \approx r - 3$  nm is the inner vesicle radius. The variance of the distribution of vesicle radii was taken to be 10 nm throughout (see above).

## Results

**Vesicle diameter.** Values of the average vesicle diameter as determined by dynamic light scattering are summarized in Table I. For each lipid, vesicles with and without incorporated enzyme were compared. In agreement with previous observations [25], protein-containing vesicles were found to have slightly larger diameters than protein-free vesicles.

**ATP-driven potassium flux.** An example of a potassium-flux measurement is shown in Fig. 2. The vesicles initially contained 140 mM  $K^+$  and 10 mM  $Na^+$  and were suspended in a medium with 10 mM  $K^+$  and 140 mM  $Na^+$ . In the presence of valinomycin a Nernst potential for  $K^+$  is established resulting in an increase of fluorescence intensity. After addition of ATP to the medium the fluorescence starts to decrease, which reflects pump-mediated extrusion of  $K^+$ .

By this method the pumping activity of reconstituted vesicles formed from different PC was studied. High transport rates were observed for di(18:1)PC, di(18:2)PC, di(20:1)PC and di(22:1)PC, whereas vesicles formed from di(18:3)PC, di(14:1)PC and di(16:1)PC were virtually inactive. It can be excluded that the di(18:3)PC, (14:1)PC and (16:1)PC vesicles were

TABLE I

AVERAGE VESICLE DIAMETER,  $d$ , FROM LIGHT-SCATTERING EXPERIMENTS

Lipid	$d$ (nm)	
	protein-free vesicles	ATPase vesicles <sup>a</sup>
Di(16:1)PC	75	116
Di(18:1)PC	72	96
Di(20:1)PC	70	90
Di(22:1)PC	72	82

<sup>a</sup> 60  $\mu$ g protein per mg lipid.

leaky to  $K^+$  since these vesicles exhibited a normal Nernst potential in the presence of valinomycin.

Potassium-flux measurements with reconstituted vesicles were carried out at different temperatures. The analysis of fluorescence transients as described in Materials and Methods yields two parameters: the average number,  $n_p$ , of pump molecules per vesicle with the ATP-binding site facing outward, and the initial turnover rate,  $v_0$ . For a given vesicle preparation the time-course of  $\Delta F/F_0$  at all temperatures could be fitted with a single value of  $n_p$  (as expected) and with variable  $v_0$ . Arrhenius plots of  $v_0$  vs.  $1/T$  are represented in Figs. 3 and 4. In Fig. 3 the single data points

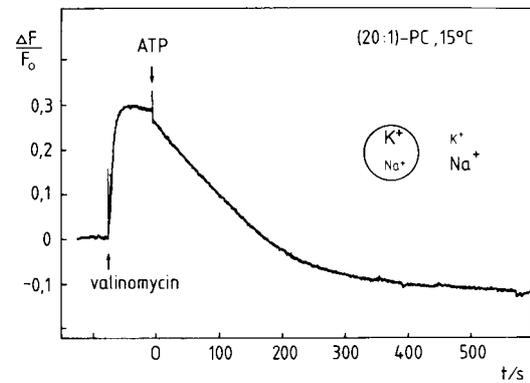


Fig. 2. ATP-driven extrusion of  $K^+$ .  $(Na^+ + K^+)$ -ATPase vesicles were formed from di(20:1)PC in buffer H containing 70 mM  $K_2SO_4$  and 5 mM  $Na_2SO_4$ . 5  $\mu$ l of the vesicle suspension (10 mg lipid/ml) were diluted into 1 ml buffer H containing 5 mM  $K_2SO_4$ , 70 mM  $Na_2SO_4$ , and 2.3  $\mu$ M Indocyanine dye. 20 nM valinomycin and 2.5 mM ATP were added successively. The fast initial signal change after ATP addition results from the dilution of the vesicle suspension.  $\Delta F/F_0$  is the relative change of fluorescence intensity, referred to the fluorescence intensity  $F_0$  prior to the addition of valinomycin. The temperature was 15°C.

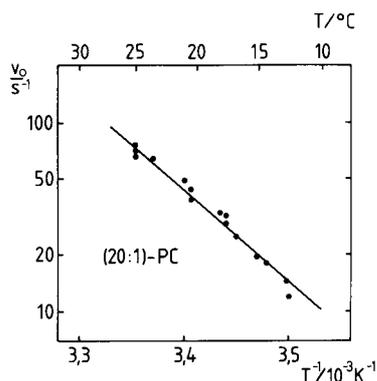


Fig. 3. Initial turnover rate,  $v_0$ , of  $(\text{Na}^+ + \text{K}^+)$ -ATPase in vesicles prepared from di(20:1)PC as a function of reciprocal temperature. The data points have been obtained from two different enzyme preparations. The slope of the line corresponds to an activation energy of  $E_a = 91$  kJ/mol.

(for di(20:1)PC) are shown in order to indicate the experimental scatter. From the slope of the line in the  $\ln v_0$  vs.  $1/T$  plot, the activation energy,  $E_a$ , is obtained using the relation  $E_a = -Rd \ln v_0/d(1/T)$ . As seen from Table II, the activation energy decreases in the order di(16:1)PC, di(18:1)PC, di(20:1)PC  $\approx$  di(22:1)PC.

In order to check for the reversibility of the temperature effects, the vesicle suspension was kept at an elevated temperature ( $37^\circ\text{C}$ ) for 30 min. Thereafter the rate of ATP-driven  $\text{K}^+$  extrusion was measured at  $27^\circ\text{C}$ . The rate was found to be the same as in an experiment at  $27^\circ\text{C}$  without pretreatment at  $37^\circ\text{C}$ .

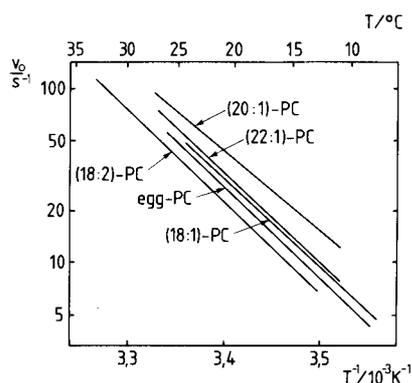


Fig. 4. Initial turnover rate,  $v_0$ , of  $(\text{Na}^+ + \text{K}^+)$ -ATPase in vesicles prepared from different phosphatidylcholines as a function of reciprocal temperature.

TABLE II

ACTIVATION ENERGY,  $E_a$ , OF ATP-DRIVEN  $\text{K}^+$  FLUX FOR VESICLES PREPARED FROM DIFFERENT PHOSPHATIDYLCHOLINES

The error limits indicate the uncertainty in determining the slope of  $\ln v_0$  vs.  $1/T$  in the Arrhenius plot.

Lipid	$E_a$ (kJ per mol)
Di(16:1)PC	(160) <sup>a</sup>
Di(18:1)PC	$115 \pm 5$
Di(20:1)PC	$91 \pm 6$
Di(22:1)PC	$100 \pm 5$
Di(18:2)PC	$103 \pm 4$
Egg PC	$103 \pm 7$

<sup>a</sup> Extrapolated value (from Fig. 6).

As mentioned above, the two rather similar phosphatidylcholines, di(16:1)PC and di(18:1)PC, yielded very different pumping activities in the reconstitution experiment. In order to study this chain-length effect further, reconstitution was carried out with mixtures of di(16:1)PC and di(18:1)PC. In Fig. 5, values of  $n_p$  and  $v_0$  are given as a function of lipid composition for a series of reconstitution experiments carried out under strictly comparable conditions, differing only in the di(16:1)PC/di(18:1)PC ratio. It is seen that  $n_p$  strongly decreases with increasing di(16:1)PC content, extrapolating to nearly zero for pure di(16:1)PC. The turnover rate,  $v_0$ , on the other hand, is rather insensitive to lipid composition. This means that the vanishing pumping activ-

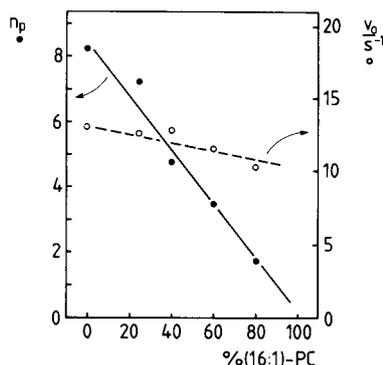


Fig. 5. Average number,  $n_p$ , of pump molecules (ATP-binding site facing outward) per vesicle and initial turnover rate,  $v_0$ , in mixtures of di(16:1)PC and di(18:1)PC. The temperature was  $16^\circ\text{C}$ .

ity of di(16:1)PC vesicles mainly results from the low yield of incorporation of (functionally oriented) pump molecules into the vesicle membrane.

The values of  $n_p$  determined from the flux rates (Fig. 5) may be compared with the maximum possible value,  $n_{p,max}$ , calculated under the assumption that the total amount of enzyme which is present at the start of the dialysis becomes incorporated into the vesicle membrane with the ATP-binding site facing outward. From the protein/lipid ratio of the solubilisate in the dialysis tube and the average vesicle radius,  $n_{p,max}$ , is estimated to be 15–20 per vesicle under the experimental conditions of Fig. 5, whereas the observed value for pure di(18:1)PC is about 8–10. This means, if half of the pump molecules are assumed to be inserted with the ATP-binding site facing outward, that about 90% of the protein initially present in the solubilisate is incorporated into the bilayer. The activation energy,  $E_a$ , of ATP-driven potassium flux as a function of composition is given in Fig. 6.  $E_a$  increases from 115 kJ/mol in pure di(18:1)PC vesicles to an (extrapolated) value of approx. 160 kJ/mol in pure di(16:1)PC vesicles.

**Rate of ATP hydrolysis.** In addition to the flux measurements, the enzymatic activity of the ATPase was determined prior to dialysis in the lipid-protein-cholesterol solubilisate. The enzymatic activity was determined at 37°C. As seen from Fig. 7, activity was small for di(16:1)PC and virtually absent for di(14:1)PC, but high for di(18:1)PC, di(20:1)PC and di(22:1)PC. In en-

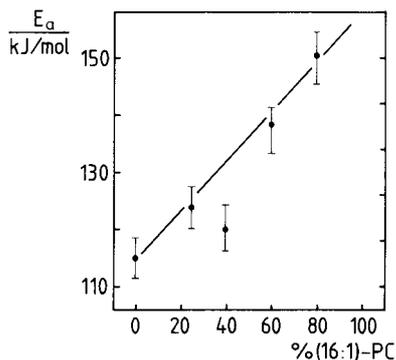


Fig. 6. Activation energy,  $E_a$ , of ATP-driven potassium flux as a function of composition in di(16:1)PC/di(18:1)PC mixtures. The error bars indicate the uncertainty in determining the slope of  $\ln v_0$  vs.  $1/T$  in the Arrhenius plot.

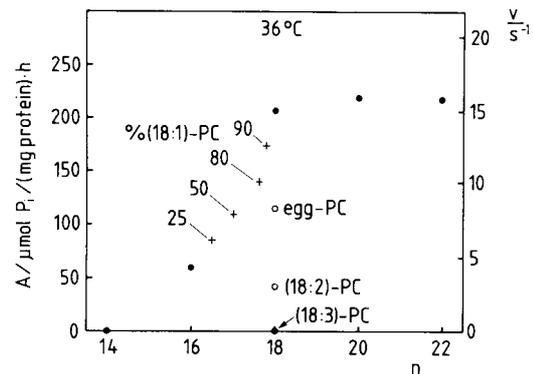


Fig. 7. Rate of ATP hydrolysis in the lipid-protein-detergent solubilisate prior to dialysis, at 36°C. 1 ml of the solubilisate (in buffer H) contained 20 mg lipid, 1.2 mg protein and 10 mg cholate. 5  $\mu$ l of this solution were added to 1 ml of the ATPase assay medium [18,20].  $n$  is the number of carbon atoms in the fatty-acid chain of the di( $n$ :1)PC. Data points between  $n = 16$  and  $n = 18$  refer to di(16:1)PC/di(18:1)PC mixtures.  $v$  is the turnover rate calculated with a molar mass of 280000 g/mol. The activity of the enzyme preparation prior to cholate solubilization was 1820  $\mu$ mol P<sub>i</sub>/(mg protein)·h.

zyme tests carried out after dialysis di(16:1)PC vesicles exhibited activity, whereas di(14:1)PC vesicles were inactive. The observed enzymatic activities thus parallel the potassium-flux rates of reconstituted vesicles, except for di(16:1)PC where transport activity was extremely low.

Increasing the number of double bonds in the hydrocarbon chain also influences the enzymatic activity. A second double bond reduces the activity to approx. 20%, a third double bond virtually abolishes activity. Egg PC which contains different fatty acid residues leads to a significant reduction of activity compared to di(18:1)PC. After dialysis part of the activity recovers. Typical enzymatic activities (in  $\mu$ mol P<sub>i</sub>/mg protein·h) are 1850 for purified enzyme, 700 after cholate solubilisation, 220 after mixing with di(18:1)PC cholate solution, and 400 after dialysis and formation of vesicles. It cannot be excluded that a fraction of the vesicles is reopened in the assay medium during incubation at 37°C.

## Discussion

In previous studies of the effects of lipid structure on the (Na<sup>+</sup> + K<sup>+</sup>)-ATPase, the rate of ATP hydrolysis has been measured after addition of

lipid to the solubilized enzyme and removal of the detergent [1–4]. Under these conditions the protein becomes associated with a newly formed lipid bilayer. By studying the enzymatic activity alone, it is not possible, however, to distinguish between enzyme molecules which are built into the bilayer in a transmembrane fashion and enzyme molecules which are merely bound to the surface of the lipid. In the experiments described above in which the rate of ATP-driven potassium transport has been measured by a fluorimetric method, only those ATPase molecules contribute to the optical signal which are incorporated into the membrane of a tight vesicle and are capable of translocating ions across the bilayer.

Determinations of the lipid contents of highly purified  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  preparations yielded lipid/protein ratios of 1 [28] or 0.8 [33]. After reconstitution of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  in vesicles using the cholate dialysis method described above the native lipid amounts to about 2% of the total lipid [34]. It is not known to what extent the native lipid exchanges with the bulk lipid of the vesicle. However, a change of the bulk lipid composition from pure di(18:1)PC to a mixture of 90/10 di(18:1)PC/di(16:1)PC results in a significant reduction of the enzyme activity (Fig. 7). This observation is consistent with the assumption that a substantial fraction of the bound native lipid exchanges with the lipid added in the reconstitution experiment.

In conventional flux studies with reconstituted vesicles, the overall transport rate, referred to the total amount of protein is measured. In this way the product of the intrinsic turnover rate,  $v_o$ , times the average number,  $n_p$ , of functional transmembrane pump molecules per vesicle (ATP-binding site facing outward) is obtained. The analysis used in our experiments allows the estimation of both factors,  $v_o$  and  $n_p$ , separately. The analysis is based on the assumption that pump molecules insert into the vesicle membrane independently so that the number of vesicles containing one, two, three, etc., pump molecules is given by Poisson statistics [12,27]. Vesicles with a relatively large number of protein molecules extrude  $\text{K}^+$  at a high rate and after a short time cease to contribute to the time-dependent fluorescence signal,  $F(t)$ . In this way the shape of  $F(t)$  becomes strongly

dependent on the average number,  $n_p$ , of outwardly oriented pump molecules per vesicle.

Using the number,  $n_p$ , of pump molecules per vesicle, the total protein content of the vesicle preparation can be determined. It was found that the fraction of functionally active enzymes is 40–45% of the initial amount of protein and more than 45% of the total protein content of the vesicle preparation. The assumption that the reconstitution procedure yields 50% inside-out oriented pump molecules [35], leads to the conclusion that 90% of the protein is in a function active form. By dialysis at lower pH values preparations of vesicles were obtained with a considerably reduced content of active enzyme. These vesicles which probably contained denatured protein exhibited a distinct leakage permeability.

Our experiments show that pumping activity of the  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  can be restored in vesicles formed from PC with (18:1), (18:2), (20:1), and (22:1) fatty-acid chains, whereas PC with shorter monounsaturated chains (16:1 and 14:1) fail to yield transport-active vesicles. These results may be compared with the observations of Johansson et al. [2] on the chain-length dependence of ATP-hydrolysis rate of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ . Their finding that enzymatic activity is low for di(14:1)PC but high for di(18:1)PC and di(20:1)PC is directly paralleled by the flux studies reported here. In the case of di(16:1)PC vesicles, however, enzymatic activity has been observed by Johansson et al. [2] (and also in our experiments), whereas transport activity is negligible. A possible explanation consists in the assumption that in the formation of di(16:1)PC vesicles, ATPase molecules become associated with the surface of the vesicle membrane but do not span the bilayer.

This assumption is supported by the results of the experiments with mixtures of di(16:1)PC and di(18:1)PC (Fig. 5). With increasing content of di(16:1)PC the average number of active pump molecules per vesicle strongly decreases, whereas the intrinsic transport rate of the pump remains almost constant (at 16°C). If the chain length of di(16:1)PC is too short to accommodate the hydrophobic portion of the ATPase [2], insertion of the protein into the bilayer may still be possible if the protein is preferentially surrounded by di(18:1)PC molecules. An influence of the lipid matrix on

pumping rate nevertheless exists, as is demonstrated by the increase of activation energy with increasing di(16:1)PC content of the membrane (Fig. 6).

The activation energy,  $E_a$ , of ion transport is highest for di(16:1)PC and decreases in the order di(18:1)PC, di(20:1)PC  $\approx$  di(22:1)PC (Table II). The values of  $E_a$  which range between 90 and 115 kJ/mol for the longer chain phosphatidylcholines may be compared with activation energies of ATP hydrolysis by the (Na<sup>+</sup> + K<sup>+</sup>)-ATPase previously reported in the literature. From experiments with kidney enzyme incorporated into artificial lipid vesicles by addition of enzyme to preformed vesicles, Kimelberg and Papahadjopoulos [5] obtained  $E_a$  values of 59 kJ/mol for di(14:0)phosphatidylglycerol (PG) and 140 kJ/mol for di(16:0)PG above the phase-transition temperature of the lipid. With isolated (Na<sup>+</sup> + K<sup>+</sup>)-ATPase from kidney an activation energy of 71 kJ/mol was observed [29]. A much higher temperature dependence of ATP hydrolysis was found for (Na<sup>+</sup> + K<sup>+</sup>)-ATPase of erythrocyte membranes [30,31], corresponding to an activation energy of  $E_a \approx 130$  kJ/mol.

Variations in the number of double bonds in the fatty-acid chain markedly affect the activity of the enzyme. In the series di(18:1)PC, di(18:2)PC, di(18:3)PC the ATP-hydrolysis rate strongly decreases (Fig. 7). The pumping rate,  $v_o$ , is (within the experimental temperature range) lower for di(18:2)PC than for di(18:1)PC (Fig. 4). Possible explanations for the effect of unsaturation on enzyme activity are a reduction of membrane thickness with increasing number of double bonds [32], and/or a decrease of membrane rigidity leading to destabilization of functional protein conformations.

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