Modulation of Na,K-ATPase and Na-ATPase Activity by Phospholipids and Cholesterol. I. Steady-State Kinetics†

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ABSTRACT: The effects of phospholipid acyl chain length (n_c), degree of acyl chain saturation, and cholesterol on Na,K-ATPase reconstituted into liposomes of defined lipid composition are described. The optimal acyl chain length of monounsaturated phosphatidylcholine in the absence of cholesterol was found to be 22 but decreased to 18 in the presence of 40 mol % cholesterol. This indicates that the hydrophobic matching of the lipid bilayer and the transmembrane hydrophobic core of the membrane protein is a crucial parameter in supporting optimal Na,K-ATPase activity. In addition, the increased bilayer order induced by both cholesterol and saturated phospholipids could be important for the conformational mobility of the Na,K-ATPase changing the distribution of conformations. Lipid fluidity was important for several parameters of reconstitution, e.g., the amount of protein inserted and the orientation in the liposomes. The temperature dependence of the Na,K-ATPase as well of the Na-ATPase reactions depends both on phospholipid acyl chain length and on cholesterol. Cholesterol increased significantly both the enthalpy of activation and entropy of activation for Na,K-ATPase activity and Na-ATPase activity of Na,K-ATPase reconstituted with monounsaturated phospholipids. In the presence of cholesterol the free energy of activation was minimum at a lipid acyl chain length of 18, the same that supported maximum turnover. In the case of ATPase reconstituted without cholesterol, the minimum free energy of activation and the maximum turnover both shifted to longer acyl chain lengths of about 22.

Although investigations on the Na,K-ATPase have been continuing for more than 40 years, important aspects of this ion-pump are still largely unknown. This includes, for example, the modulation of the catalytic activity of the Na,K-ATPase by plasma membrane lipids into which it is suspended.

According to the fluid mosaic membrane concept of Singer and Nicolson (*1*), the proteins partition into the lipid bilayer floating in a pseudo two-dimensional medium of fluid lipids. Bilayer fluidity is a crucial property of the cell membrane and ensures any necessary lateral and conformational mobility of the proteins. However, fluidity does not necessarily imply randomness. Membranes are in fact highly structured fluids separated into lipid domains important for the differential functions of the various proteins (*2*).

Relatively few systematic investigations of the specific lipid dependency on Na,K-ATPase function exist (cf. ref *3*), and an understanding of the protein/lipid interaction in general is still missing. However, several studies have documented lipid effects on specific characteristics of the Na,K-ATPase mechanism. These include effects of phospholipid acyl chain length, n_c (*4*), saturation (*5*), and phospholipid headgroup (*6*, *7*), as well as of cholesterol (*8*−*10*). One important question to address is whether protein/lipid interaction is indirect via, for example, order and fluidity, or more specific, implying a closer interaction or even binding between certain lipids and the Na,K-ATPase. The very complex lipid composition of the plasma membrane with lipid domain formation is probably very important in the regulation of Na,K-ATPase, for example, by protein kinases (*11*, *12*), but does it have any bearing on the Na,K-ATPase activity per se? In the present investigation, these questions are addressed using reconstitution of highly purified Na,K-ATPase into liposomes of defined lipid composition (*7*). More specifically, the effects of phospholipid acyl chain length, its degree of saturation, and of cholesterol on the steady-state catalytic activity of reconstituted Na,K-ATPase purified from shark rectal glands are investigated. In a following paper, the issue of lipid effects on some partial reactions investigated by initial rate measurements will be addressed (Cornelius, unpublished results).

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Membrane bound Na,K-ATPase (EC 3.6.1.37) from rectal glands of the shark *Squalus achantias* was prepared as previously described (*13*). The specific hydrolytic activity measured at 37 °C was 30−33 U/mg of protein at standard conditions (120 mM Na^+, 30 mM K^+, 4 mM Mg^{2+}, 3 mM ATP, and 30 mM histidine, pH 7.5) according to Ottolenghi (*14*). The protein content was determined according to Lowry et al. (*15*) using bovine serum albumin as standard. The turnover number (k_{cat}) is calculated from the specific activity by dividing by the number of phosphorylation sites, which was 2.5 nmoles/mg of protein determined according to Cornelius (*16*).

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Reconstitution. Functional reconstitution of shark Na,K-ATPase was achieved as previously described (*7*, *17*): initially membrane bound Na,K-ATPase was solubilized using the nonionic detergent $C_{12}E_8$. Next, the lipids of choice were solubilized using the same detergent, and the two solutions were mixed at a protein/lipid weight ratio estimated to give a final ratio of 1:20. Ample mixing time is required to ensure proper equilibration of the protein/lipid/detergent mixed micelles (*3*) and to allow exchange of the motional restricted annular lipids that coat the protein surface to exchange with the bulk lipids. The latter process has been shown to take place on a microsecond time scale (*18*). When the detergent was subsequently removed by addition of hydrophobic bio-beads, liposomes containing reconstituted Na,K-ATPase spontaneously formed. Careful control during enzyme solubilization ensures that this reconstitution took place without loss of catalytic activity or ion-transport capacity (*7*). Unless otherwise stated, the proteoliposomes were produced in 130 mM NaCl, 2 mM $MgCl_2$, and 30 mM histidine, pH 7.0.

The proteoliposomes produced were unilamellar and large with a diameter of about 100 nm, as determined from freeze-fracture EM pictures and quasi-elastic laser light scattering (*19*). Before they can be used for quantitative experiments they were thoroughly characterized including determination of the pump density (protein content and size distribution) and symmetry of protein insertion since several of these parameters depended on the lipid composition, as described below.

Protein determination of reconstituted ATPase was performed according to the Peterson modification (*20*) of the Lowry method. In this, the reconstituted protein was quantitatively precipitated with sodium deoxycholate and trichloroacetic acid followed by re-suspension in water. SDS was included in the copper tartrate solution to leave the lipid transparent and noninterfering. Bovine serum albumin was run as standard.

After reconstitution, the hydrolytic activity of Na,K-ATPase originates from the turnover of enzyme molecules with exposed ATP-sites, i.e., molecules with their cytoplasmic side facing the outside. In principle, these include inside-out (i/o) oriented enzyme that has the ATP site exposed to the medium as well as Na,K-ATPase reconstituted with both sides exposed (nonoriented enzyme, (n/o)) that can be activated by addition of ATP and inhibited by the addition of ouabain, whereas right-side out (r/o) enzymes have their substrate sites shielded inside the liposomes. The n/o fraction of Na,K-ATPase represents reconstituted enzyme in the sense that it is found associated with the lipid phase of the proteoliposomes (*7*). It may represent very leaky vesicles or externally adsorbed enzyme (*3*). n/o-oriented Na,K-ATPase hydrolyses ATP added to the medium but performs no vectorial cation transport.

For each proteoliposome preparation the fractions of r/o, i/o, and n/o-oriented enzyme were determined from functional tests as previously described in detail (*7*, *17*). Essentially, the fraction of i/o-oriented Na,K-ATPase was estimated from the fractional activation of hydrolysis by internal (extracellular) K^+ in the presence of external ouabain.

ATPase Activity. The rate of ATP-hydrolysis of reconstituted Na,K-ATPase was measured using [^{32}P]ATP as described in ref *21*. Measurement of maximum hydrolytic capacity of i/o-oriented Na,K-ATPase was performed after preincubation of the proteoliposomes with Mg^{2+} (5 mM), P_i (1 mM), and ouabain (1 mM) to inhibit ATP-hydrolysis of n/o enzyme. The hydrolytic Na,K-ATPase activity was determined in a test medium containing: 50 μM ATP, 130 mM NaCl, 10 mM KCl, and 1 mM $MgCl_2$, and 0.7 μM nigericin was included in the test medium to ensure rapid equilibration of K^+ across the proteoliposomes (*16*). Na-ATPase activity was determined in the absence of K^+ in the test medium. The lower ATP concentration used to obtain a reasonable specific radioactivity in the assay of hydrolytic activity of reconstituted ATPase is near-saturating since the apparent ATP affinity for the Na,K-ATPase and Na-ATPase reactions is ~10 and 0.2 μM, respectively [(*21*) and the present investigation, not shown]. The turnover number was calculated by dividing the specific activity with the number of phosphorylation sites, which after reconstitution of Na,K-ATPase was found to be up to 6.8 nmoles/mg of protein depending on the lipid composition, close to the theoretical maximum number of phosphorylation sites assuming one site per $\alpha\beta$-monomer (*16*).

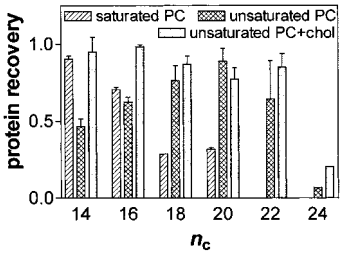


FIGURE 1: Total protein incorporation into liposomes after reconstitution of Na,K-ATPase with either saturated PC or monounsaturated PC with and without 40 mol % cholesterol at increasing acyl chain length (n_c). For saturated PC the protein recovery decreased with increasing acyl chain length, whereas for monounsaturated PC the optimal acyl chain length was 20. The presence of cholesterol together with monounsaturated PC increased the total protein incorporation preferentially at the shorter acyl chain lengths.

Materials. Highly purified phospholipids were obtained from Avanti Polar Lipids. Cholesterol was from Sigma. ATP, purchased as the sodium salt from Boehringer Mannheim, was converted to the Tris salt by chromatography on a Dowex 1 column (Sigma). γ^{32}P-ATP was from Amersham. Nigericin was from Molecular Probes.

RESULTS

Reconstitution. For comparison of lipid effects on reconstituted Na,K-ATPase, several parameters must be controlled: For one, the protein/lipid ratio of the proteoliposomes is important for the specific hydrolytic activity of reconstituted Na,K-ATPase (*7*). It is, therefore, important that Na,K-ATPase reconstituted into liposomes with different lipid compositions is performed to a near identical protein/lipid ratio. This is not a trivial matter since the amount of reconstituted protein depends on the lipid properties (e.g., phase and acyl chain length). This is illustrated in Figure 1 where protein recovery after reconstitution into saturated or monounsaturated PC of increasing acyl chain lengths, n_c, from 14 to 24 is shown. As indicated, reconstitution of Na,K-ATPase with saturated PC results in a decreased protein recovery in the proteoliposomes both when n_c increases and compared to monounsaturated PC. For saturated PC protein

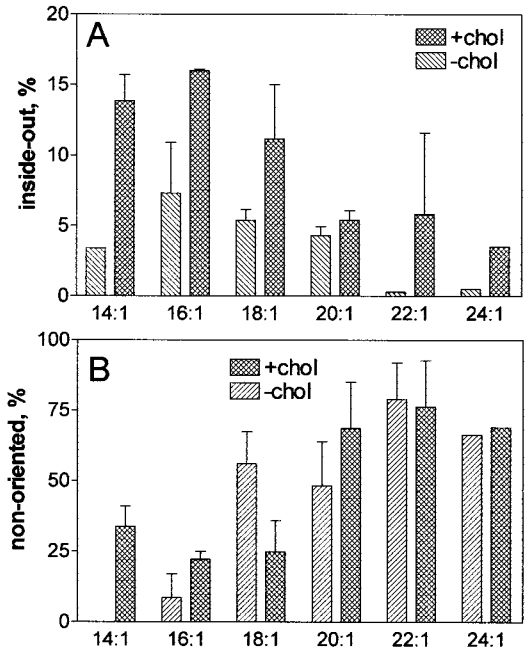


FIGURE 2: Orientation of Na,K-ATPase reconstituted with monounsaturated PC of increasing acyl chain length in the absence or presence of 40 mol % cholesterol. Panel A shows the fraction of i/o-oriented Na,K-ATPase in the liposomes. Independent of cholesterol the i/o-orientation is maximal at an acyl chain length of 16. Panel B shows the fraction of n/o-oriented Na,K-ATPase, which increases significantly at increasing acyl chain length both with and without cholesterol.

recovery is optimal at an acyl chain length of 16, whereas for monounsaturated PC the optimal protein recovery is at $n_c = 20$. At $n_c > 22$, protein recovery decreased probably as a result of the unfavorable bilayer thickness and/or the higher phase transition temperature. As also shown in Figure 1, inclusion of 40 mol % cholesterol together with monounsaturated PC considerably increased the protein recovery in the proteoliposomes especially at the shorter acyl chain lengths. Therefore, to compare lipid effects on reconstituted Na,K-ATPase it is necessary to adjust the initial protein/lipid ratio in the mixed protein/lipid/detergent micelle solution to obtain a fixed protein/lipid ratio in the proteoliposomes after removal of the detergent. Second, the protein orientation in the proteoliposomes also depends on the acyl chain length of the phospholipid used for reconstitution. As seen from Figure 2 the insertion of enzyme after reconstitution is asymmetric, as previously found with $C_{12}E_8$ (*17*). The fraction of inside-out Na,K-ATPase is optimum for monounsaturated PC of $n_c = 16$. Finally, inclusion of 40 mol % cholesterol together with PC doubled the i/o-fraction at all lipid acyl chain lengths, except for $n_c = 20$ (Figure 2A). Since only Na,K-ATPase incorporated with an inside-out orientation are activated upon ATP addition to the medium it is imperative for each lipid composition to determine the fraction of i/o oriented ATPase to ascribe the measured hydrolytic activity to the population of active pumps (i.e., the specific activity or turnover). Right-side out pumps are devoid of substrate (ATP is impermeable) and incubation of the proteoliposomes with MgP_i and ouabain is used to inhibit any enzyme that is not fully incorporated or only adsorbed to the liposomes (nonoriented, n/o).

Lipid Fluidity. Fluidity is an essential feature of lipids and important for supporting optimal activity of integral proteins

Table 1: Main Phase Transition Temperature (T_m) of Various Saturated and Mono-unsaturated PC (collected in ref *58*) and Hydrophobic Thickness (d) of Bilayer in the Liquid-Crystalline Phase Calculated from the Equations $\langle d_{sat} \rangle \cong 1.75(n_c - 1)$ and $\langle d_{unsat} \rangle \cong 1.75(n_c - 2.6)$

lipid	d (Å)	T_m (°C)	lipid	d (Å)	T_m (°C)
di-C14:0 PC	22.8	24	di-C14:1 PC	20.0	
di-C16:0 PC	26.3	41	di-C16:1 PC	23.4	−36
di-C18:0 PC	29.8	55	di-C18:1 PC	27.0	−19
di-C20:0 PC	33.3	66	di-C20:1 PC	30.4	−4
			di-C22:1 PC	34.0	13
			di-C24:1 PC	37.4	27

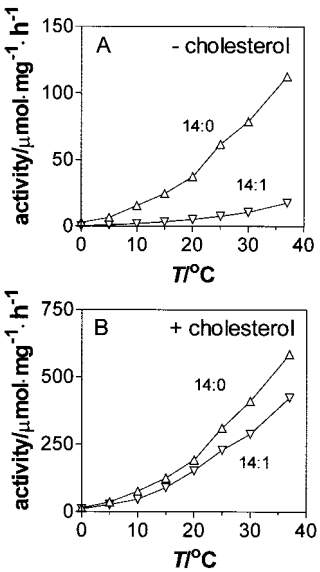


FIGURE 3: Temperature sensitivity of ATPase activity of Na,K-ATPase reconstituted with di-C14:0 PC or di-C14:1 PC either without cholesterol (panel A) or in the presence of 40 mol % cholesterol (panel B). Means of three determinations are indicated. SEM are less than the size of the symbols.

such as the Ca-ATPase (*22*−*24*). In the cell membrane, fluidity is ensured by a certain proportion of unsaturated phospholipid acyl chains and/or by the presence of cholesterol. Monounsaturated PC with chain length lower than 24 all have a main phase transition temperature, T_m, below room temperature, whereas saturated PC with longer chain lengths than 14 have T_m well above room temperature (see Table 1). This makes them difficult to use for reconstitution of Na,K-ATPase since incorporation into a gel-phase is difficult and an increase of temperature may be deleterious to the enzyme.

Figure 3 compares the temperature sensitivity of Na,K-ATPase activity for Na,K-ATPase reconstituted with either di-C14:0 PC[1] or di-C14:1 PC. As demonstrated in the upper panel, the Na,K-ATPase activity of Na,K-ATPase reconstituted with saturated di-C14:0 PC is higher than with monounsaturated PC of the same acyl chain length, even at temperatures below 25 °C where di-C14:0 PC is in the gel phase. This indicates that lipid fluidity is not the most important property and that other parameters are significant

[1] Abbreviations: di-Cn_c:1 PC and di-Cn_c:0 PC, phosphatidylcholine with unsaturated or saturated lipid acyl chains, respectively, of lengths n_c; $C_{12}E_8$, octaethylene dodecylmonoether; TIDPC/16, 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-(tributylstannyl)-4-(trifluromethyl-3*H*-diazirin-3-yl)-benzyl]oxy]carbonyl]nonanoyl]-*sn*-glycero-3-phosphocholine.

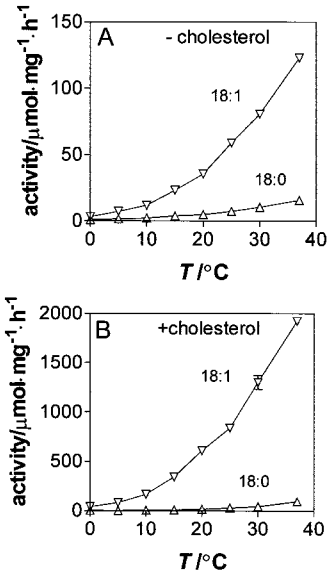


FIGURE 4: Temperature sensitivity of ATPase activity of Na,K-ATPase reconstituted with di-C18:0 PC or di-C18:1 PC in the absence of cholesterol (panel A) or together with 40 mol % cholesterol (panel B). Means of three determinations with SEM indicated are shown.

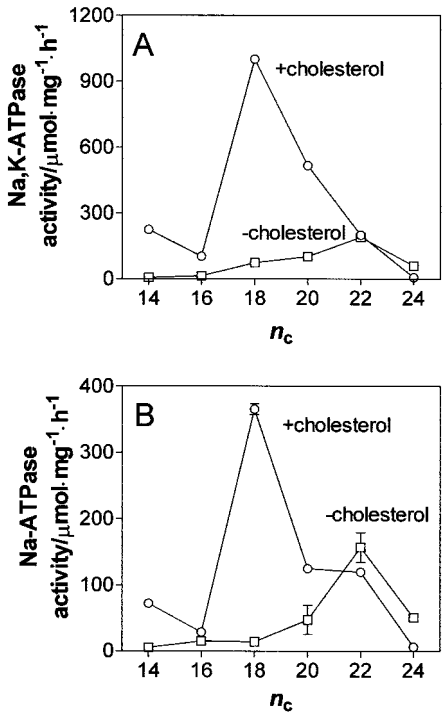


FIGURE 5: Na,K-ATPase activity (panel A) or Na-ATPase activity (panel B) of Na,K-ATPase reconstituted with monounsaturated PC with acyl chains lengths (n_c) increasing from 14 to 24 in the presence (○) or absence (□) of 40 mol % cholesterol. The activity was measured at 25 °C, which is above the main transition temperature for all PC used; however, equivalent curves were obtained at all temperatures employed. Without cholesterol and at $n_c \geq 22$, the Na-ATPase activity and the Na,K-ATPase activity were equal, i.e., no activation of turnover by extracellular (internal in liposomes) K^+ was observed.

in supporting optimal Na,K-ATPase activity. The lower panel demonstrates that inclusion of 40 mol % cholesterol together with PC increases the Na,K-ATPase activity for both preparations, but most for the monounsaturated PC resulting in about identical hydrolytic activities for saturated and monounsaturated PC.

di-C14:0 PC proved to be the only saturated phospholipid that supported this high activity of reconstituted Na,K-ATPase. The hydrolytic activity of Na,K-ATPase reconstituted with di-C18:0 PC, for example, supported only 1 and 5% as compared to di-C18:1 PC in the absence and presence of 40 mol % cholesterol, as seen from Figure 4. Reconstitution of Na,K-ATPase with saturated PC of acyl chain length 16, 18, or even 20 is still possible as seen from Figure 1, although the temperature at which reconstitution is performed (25 °C) was well below the phase transition for these phospholipids (see Table 1). However, the orientation of the reconstituted enzyme appears to be mainly n/o, whereas the fractions of i/o-oriented enzyme were too low to be measured accurately with the present methods. This is not because measurement of such low activities constitutes any problem, but because when the n/o fraction is high the fraction of i/o must be estimated from the difference between two almost identical quantities (*7*, *17*).

Hydrophobic Thickness. One other property of the lipid bilayer that must have obvious importance for the activity of an integral enzyme is its thickness. In a lipid bilayer of phosphatidylcholine (PC) the effective hydrophobic thickness, $\langle d \rangle$ (in Å) is determined approximately by the acyl chain length, n_c. For saturated PC, the relation $\langle d_{sat} \rangle \cong 1.75(n_c - 1)$ applies (*25*). Double bonds in the acyl chains reduce the hydrophobic thickness so that for monounsaturated PC the semiempirical relation applies $\langle d_{unsat} \rangle \cong 1.75(n_c - 2.6)$, i.e., di-C14:0 PC and di-C16:1 PC have about the same hydrophobic thickness.

Another important plasma membrane component is cholesterol. Most animal plasma membranes contain 30−40 mol % cholesterol. Cholesterol increases the acyl chain order of phospholipid (*26*). Cholesterol >25 mol % promotes formation of the liquid-ordered phase, which is fluid from the point of view of lateral disorder and diffusion, but at the same time the acyl chains are highly ordered (*26*). Therefore, apart from influencing the fluidity, cholesterol also increases the thickness and the physical strength of the plasma membrane considerably, without limiting the lateral mobility of integral proteins.

In Figure 5A the temperature dependency of the hydrolytic activity of shark Na,K-ATPase reconstituted into liposomes of monounsaturated PC of increasing acyl chain length from $n_c = 14$ to 24 is shown together with similar data where reconstitution was performed with the inclusion of 40 mol % cholesterol together with PC. Comparing the two conditions, three results are immediately apparent: First, cholesterol shifted the optimal acyl chain length from 22 to a shorter length of $n_c = 18$. Second, cholesterol increased significantly the maximum specific activity or the turnover of the pump for acyl chain lengths, $n_c < 22$. Finally, the maximum turnover obtained with di-C18:1 PC + 40 mol % cholesterol matched the turnover measured in natural membranes (see Discussion).

Na-ATPase Activity. In the physiological mode of the Na,K-ATPase reaction 3 cytoplasmic Na^+ are expelled and 2 extracellular K^+ taken up for each ATP split (*3*). The extracellular K^+ can be replaced by the K^+-congeners Rb^+, Tl^+, and Cs^+, and even by Na^+. In the latter case, a 3:2 Na^+:Na^+-exchange takes place that is electrogenic and requires ATP hydrolysis (*27*, *28*). The catalytic activity associated

Table 2: Rate Constants for Shark Na,K-ATPase Measured at 20 °C for the Reactions Depicted in Scheme 1 (*47*)

rate constant	Na,K-ATPase	Na-ATPase
$k_1/M^{-1}\,s^{-1}$	8×10^5 [a]	4×10^7 [a]
k_{-1}/s^{-1}	$\sim 2-10$ [b]	$\sim 2-10$ [b]
k_2/s^{-1}	525	525
k_3/s^{-1}	100	100
k_{-3}/s^{-1}	30	30
k_4/s^{-1}	$\sim 1.5 \times 10^3$ [c]	2[d]

[a] Estimated from k_{-1} by dividing with an equilibrium binding constants K_D for ATP of 0.1−0.2 μM (*59*, *60*) and from the 50 times smaller apparent ATP-affinity in the presence of K^+. [b] From ref *51*. [c] Estimated from a measured combined rate constant of $\sim 100\ s^{-1}$ measured at 0 °C and a Q_{10} of ∼4 (*16*). [d] From ref *28*.

with this reaction is termed Na-ATPase activity. Under physiological conditions, extracellular K^+ binds to the E_2P phosphoform that leads to a rapid dephosphorylation and occlusion of K^+. When Na^+ replaces extracellular K^+-binding the subsequent dephosphorylation becomes slow, about 1−5 s^{-1} at 22 °C (*28*) and is believed to become rate limiting (Table 2).

In the following, it was investigated how the steady-state Na-ATPase activity was affected by phospholipids and cholesterol compared to the Na,K-ATPase activity. In Figure 5B, the Na-ATPase activity is shown after reconstitution with monounsaturated phosphatidylcholine of increasing acyl chain length in the presence and absence of 40 mol % cholesterol. As demonstrated the same general picture was seen as for Na,K-ATPase activity with a shift of the optimum n_c from 22 to 18 when cholesterol was present. Also noted from Figure 5 is that almost no K^+-activation is observed after reconstitution of Na,K-ATPase with di-C22:1 PC and di-C24:1 PC in the absence of cholesterol, i.e., the Na,K-ATPase and the Na-ATPase activities were identical. In the presence of cholesterol, the K^+-activation was reestablished but to a much lower extent than at shorter lipid acyl chain lengths.

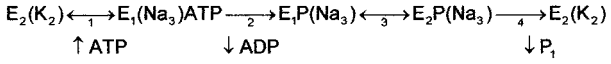
Activation Energy. According to transition state theory the rate constant, k, for an elementary chemical reaction follows the equation,

$$k = \frac{k_B T}{h} \exp\frac{\Delta S^{\ddagger}}{R} \exp\frac{-\Delta H^{\ddagger}}{RT} \qquad (1)$$

where k_B is the Boltzmann constant, h is the Planck constant, R is the gas constant, T is the temperature, $\Delta S^{\ddagger}$ is the entropy of activation, and $\Delta H^{\ddagger}$ is the enthalpy of activation. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be determined from the slope ($-\Delta H^{\ddagger}/R$) and intercept ($\Delta S^{\ddagger}/R + 23.76$) on the $\ln(k_{cat}/T)$-axis of the linear plot of $\ln(k/T)$ versus $1/T$, the Eyring plot. From these values, the free energy of activation, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ can be calculated. A detailed study of the temperature dependence of the enzyme-catalyzed reaction thus requires that the individual rate constants be separated. Eyring plots of steady-state velocity (v), or turnover number (k_{cat}) versus temperature may also be linear, which is usually taken to indicate that over the temperature range investigated a single rate constant in the reaction cycle is rate determining. Curvatures in Eyring plots are usually interpreted from complex kinetics where rate-limitation shifts between steps in the reaction or from changes in the structure of the enzyme.

The overall Na,K-ATPase reaction cycle is frequently described by the Albers-Post scheme (*29*, *30*). The catalytic cycle can be simplified to the following reactions (Scheme 1):

Scheme 1



The steps 2 and 4 are irreversible since the concentrations of ADP and P_i are essentially zero at the experimental conditions used. The experimentally determined rate constants relating to this model is given in Table 2 for shark enzyme at 20 °C. In the physiological Na,K-ATPase reaction, the $E_2 \rightarrow E_1$ conformational transition associated with K^+-deocclusion is accelerated considerably by ATP (*31*). At the near-saturating ATP-concentration used (50 μM), this step is partially rate determining together with the $E_1P \rightarrow E_2P$ reaction. However, in the absence of K^+ the apparent ATP affinity increases significantly and the dephosphorylation step becomes very slow and rate limiting for the Na-ATPase reaction. Although the second-order rate constant k_{cat} is composite, it will be determined mainly by k_1 and k_3 for the Na,K-ATPase reaction, whereas in the Na-ATPase reaction it will be dominated by k_4. Thus, Eyring plots of k_{cat} versus temperature should tend to be linear for the Na-ATPase reaction and also for the Na,K-ATPase reaction provided that the temperature sensitivity is similar for the two conformational transitions $E_2 \rightarrow E_1$ and $E_1P \rightarrow E_2P$.

In Figure 6A,B the temperature dependence of Na,K-ATPase activity (Figure 6A) or Na-ATPase activity (Figure 6B) given as the turnover number, k_{cat}, of native membrane-bound Na,K-ATPase is shown. Because of the irreversible thermal inactivation of Na,K-ATPase the turnover numbers at 25, 30, and 37 °C were obtained from extrapolations to zero time indicated in the figures by a broken regression line. The inset shows the Eyring plots of the data that appear linear. From the plots $\Delta H^{\ddagger}$ for Na,K-ATPase activity was found to be 76.3 ± 1.9 kJ/mol, and $\Delta S^{\ddagger}$ was found to be 46.6 ± 2.3 J/K·mol. For Na-ATPase activity a lower value for $\Delta H^{\ddagger}$ of 60.1 ± 1.0 kJ/mol was found, and the entropy of activation, $\Delta S^{\ddagger}$, becomes negative, −32.0 ± 0.6 J/K·mol. From these values, the free energy of activation at 25 °C can be calculated to 62.4 ± 1.6 kJ/mol for Na,K-ATPase activity, and 69.6 ± 1.2 kJ/mol for Na-ATPase activity. A lower activation energy for Na-ATPase (∼64 kJ/mol) than for Na,K-ATPase (∼89 kJ/mol) has previously been found for shark Na,K-ATPase (*32*).

In Figure 7 A,B Eyring plots of Na,K-ATPase reconstituted with di-C18 PC in the absence and presence of cholesterol are shown comparing Na,K-ATPase activity and Na-ATPase activity. As demonstrated, the enthalpy of activation for Na,K-ATPase and Na-ATPase (slopes of the Eyring plots) are identical, $\Delta H^{\ddagger} \sim 68$ kJ/mol in the absence of cholesterol (Figure 7A) and both increased to the same extent of $\Delta H^{\ddagger} \sim 75$ kJ/mol in the presence of 40 mol % cholesterol together with monounsaturated PC (Figure 7B). For both Na,K-ATPase activity and Na-ATPase activity $\Delta S^{\ddagger}$ is found to be negative in the absence of cholesterol (−10 and −14 J/K·mol, respectively), but positive in the presence of cholesterol (59 and 54 J/K·mol, respectively). Thus, the free energy of activation for both the Na-ATPase and the

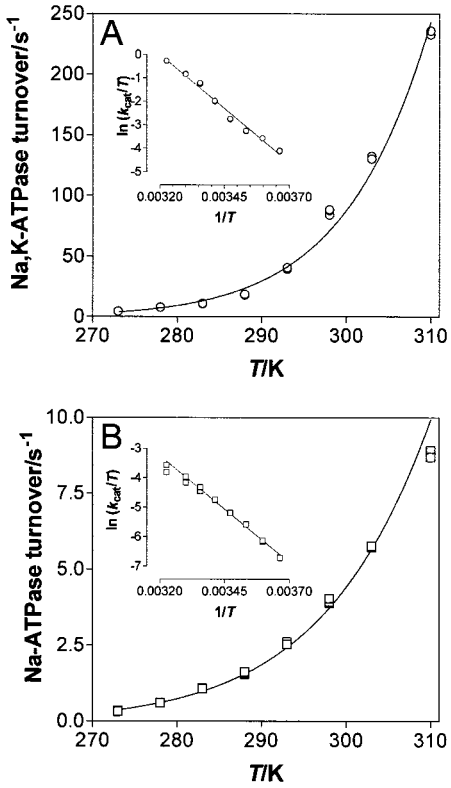


FIGURE 6: Temperature dependence of membrane bound Na,K-ATPase in the presence of 20 mM K^+ (Na,K-ATPase activity, panel A) or in the absence of K^+ (Na-ATPase activity, panel B). The curves are fit to the steady-state turnover number (k_{cat}) using the Eyring equation (eq 1). The insets show Eyring plots of the data. The values at 25, 30, and 37 °C were obtained from zero time extrapolations. The fitting parameters (slope $= -\Delta H^{\ddagger}/R$ and intercept on the $\ln(k_{cat}/T)$-axis $= \Delta S^{\ddagger}/R + 23.76$) gave the following values for Na,K-ATPase activity: $\Delta H^{\ddagger} = 76.3 \pm 1.9$ kJ/mol and $\Delta S^{\ddagger} = 46.6 \pm 1.3$ J/K·mol. For Na-ATPase: $\Delta H^{\ddagger} = 60.1 \pm 1.0$ kJ/mol and $\Delta S^{\ddagger} = -32.0 \pm 0.6$ J/K·mol. Thus, the free energy of activation at 25 °C, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, was 62 and 70 kJ/mol, respectively. The turnover number is calculated by dividing the specific activity with a site no. of 2.5 nmol/mg, i.e., at 25 °C the specific activities are 642.2 and 30.3 μmol·mg^{-1}·h^{-1} for the Na,K.ATPase and Na-ATPase reaction, respectively.

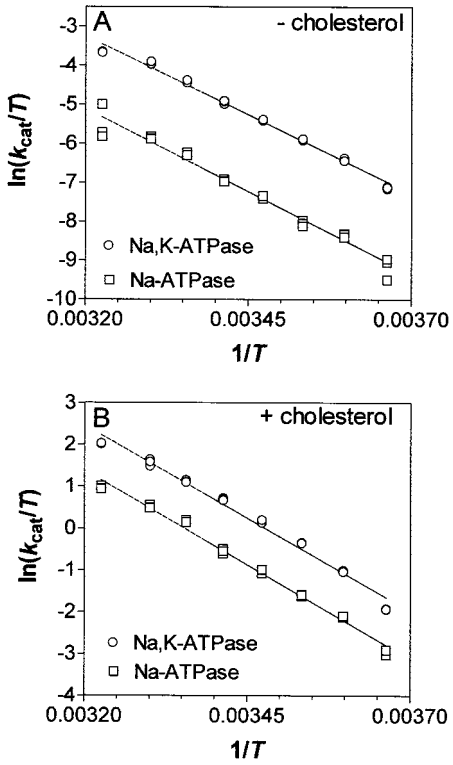


FIGURE 7: Eyring plots of Na,K-ATPase turnover (○) and Na-ATPase turnover (□) for Na,K-ATPase reconstituted with di-C18:1 PC in the absence (panel A) or presence of 40 mol % cholesterol (panel B). The straight lines are linear regression curves. In the absence of cholesterol, good linear relationships are found, whereas in the presence of cholesterol the results deviate from linearity at the higher temperatures. From the slopes of the lines the enthalpy of activation for Na,K-ATPase activity can be calculated to $\Delta H^{\ddagger} = 67.0 \pm 1.4$ kJ/mol and 73.7 ± 1.5 kJ/mol in the absence and presence of cholesterol, respectively. For Na-ATPase activity, the equivalent numbers are 70.9 ± 2.5 and 74.9 ± 1.5 kJ/mol in the absence and presence of cholesterol. $\Delta S^{\ddagger}$ obtained from the intersect of the lines with the ordinate is for Na,K-ATPase in the absence of cholesterol: -9.9 ± 0.2 J/K·mol and in the presence of cholesterol: 58.8 ± 1.5 J/K·mol. Thus, the free energy of activation at 25 °C, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ becomes 70.0 ± 1.5 and 56.2 ± 1.4 kJ/mol for the Na,K-ATPase reaction in the absence and presence of cholesterol, respectively. For Na-ATPase activity the calculated values for $\Delta S^{\ddagger}$ are -14.3 ± 0.7 and 54.0 ± 1.1 J/K·mol in the absence and presence of cholesterol, respectively. $\Delta G^{\ddagger}$ then becomes 74.8 ± 2.7 and 58.8 ± 1.2 kJ/mol in the absence and presence of cholesterol, respectively, at 25 °C. A typical experiment in triplicate is shown.

Na,K-ATPase reaction decreased by about 15 kJ/mol in the presence of cholesterol from about 72 kJ/mol to about 57 kJ/mol.

If the fraction of nonoriented enzyme (n/o) in the proteoliposomes was not inhibited by preincubation with MgPi + ouabain (see Materials and Methods) the Eyring plots became curved (not shown). This indicates that the n/o-fraction of enzyme is only partially embedded into the bilayer and differs kinetically from the i/o-orientated enzyme, which is fully incorporated into the bilayer.

For longer acyl chain lengths than $n_c = 20$, $\Delta S^{\ddagger}$ dropped to negative values also in the presence of cholesterol and became equal to the values in the absence of cholesterol. The calculated thermodynamic parameters were, however, more uncertain at the longest lipid acyl chain lengths due to an increased tendency for curvature in the Eyring plots.

From the temperature dependence of Na,K-ATPase activity and Na-ATPase activity the thermodynamic quantities were calculated and depicted as a function of the lipid acyl chain length of monounsaturated PC in the presence and absence of 40 mol % cholesterol (Figure 8). As indicated in Figure 8A, the enthalpy of activation for the two reactions, Na,K-ATPase and Na-ATPase, seemed to vary in a similar fashion as a function of increasing lipid acyl chain length, and both being higher by about 10−15 kJ/mol in the presence of cholesterol. The entropies of activation are shown in Figure 8B. Negative entropies of activation were found in the absence of cholesterol for both Na,K-ATPase and Na-ATPase at all lipid acyl chain lengths, whereas in the presence of 40 mol % cholesterol $\Delta S^{\ddagger}$ increased and gave positive values for lipid acyl chain lengths up to 20. Both the enthalpy of activation and the entropy of activation were more or less constant for acyl chain lengths up to 20 and decreased abruptly at $n_c \geq 22$. Cholesterol increased both the enthalpy of activation and the entropy of activation resulting in a decreased free energy of activation (Figure 8C). This effect of cholesterol was reduced, however, with increasing phospholipid acyl chain length resulting in almost identical $\Delta G^{\ddagger}$ at $n_c \geq 22$. With cholesterol $\Delta G^{\ddagger}$ is minimal at an acyl chain

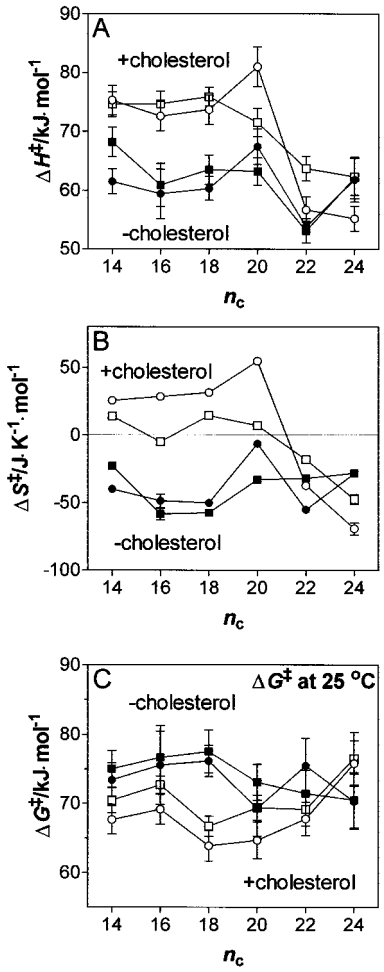


FIGURE 8: Calculated thermodynamic quantities for Na,K-ATPase reconstituted with monounsaturated PC as a function of acyl chain length (n_c). Values for Na,K-ATPase activity are indicated by circles (○, ●) and values for Na-ATPase activity by squares (□, ■). In all cases the data without cholesterol is indicated by filled symbols and the data with 40 mol % cholesterol with open symbols. Upper panel shows $\Delta H^{\ddagger}$, middle panel $\Delta S^{\ddagger}$, and lower panel free energy of activation, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$. The data are means ± SEM of three independent experiments each run in triplicate.

length of 18, whereas in the absence of cholesterol the minimum $\Delta G^{\ddagger}$ is shifted to an acyl chain length of 22.

DISCUSSION

In this study, the effects of phospholipids and cholesterol on the steady-state Na,K-ATPase and Na-ATPase hydrolytic activity were investigated by reconstitution of the ATPase into liposomes of defined lipid composition. For phospholipids, the effects of acyl chain length and degree of saturation on the steady-state ATPase activity were specifically studied. Apart from the effects on catalytic activity, phospholipids and cholesterol were also found to influence a number of parameters regarding reconstitution itself. This included the symmetry of protein insertion and the amount of protein recovered in the liposomes (Figures 1 and 2). Therefore, these parameters were controlled and taken into account to compare the specific effects of phospholipids and cholesterol on the ATPase activity per se.

Since membrane fluidity is considered essential for activity of integral enzymes it was somewhat surprising that saturated di-C14:0 PC supported higher Na,K-ATPase activity than monounsaturated di-C14:1 PC (Figure 3) even at temperatures below its T_m (24 °C, Table 1). Comparable experiments with Ca-ATPase (*23*) have previously shown no detectable activity below T_m when the Ca-ATPase was reconstituted with di-C14:0 PC. Furthermore, reconstitution of Na,K-ATPase could be demonstrated using saturated PC at even longer acyl chain length of 16, 18, or 20 (Figure 1) and higher T_m of 41, 55, and 66 °C (Table 1), respectively, although they all supported very low hydrolytic activities (cf. Figure 4). Finally, using such phospholipids resulted in reconstitution with a lower protein recovery and the Na,K-ATPase was found to be inserted with a very high fraction of n/o enzyme. It is known that protein can become immiscibile in gel phase phospholipids (*33*) and it is possible that in such conditions a major fraction of Na,K-ATPase rather than transmembrane inserted into the bilayer just adsorbs to it.

For monounsaturated PC, a clear dependence of specific hydrolytic activity on fatty acyl chain length was found (Figures 4 and 5). Without cholesterol, the phospholipid supporting optimum activity is di-C22:1 PC equivalent to a hydrophobic thickness of $d \sim 34$ Å (Table 1). Inclusion of 40 mol % cholesterol together with PC increased the maximum hydrolytic activity significantly for all phospholipids with acyl chain lengths lower than 22 and shifted the optimum to phospholipids with acyl chain length of 18 equivalent to a hydrophobic thickness of $d \sim 27$ Å (Table 1). This was found to be the case both at physiological conditions (Na,K-ATPase) and in the absence of K^+ (Na-ATPase). Cholesterol is known to increase lipid acyl chain order (*26*) and bilayer thickness (*34*). This could explain the increased optimal phospholipid acyl chain length found in the absence of cholesterol (Figure 5): A shift in optimal acyl chain lengths from 22 to 18 corresponds to a shift in hydrophobic thickness of the bilayer from 34 to 27 Å, i.e., a 21% decrease. Such a decrease is in accordance with what cholesterol is expected to counterbalance by increasing bilayer thickness (*33*). Therefore, since all monounsaturated PC with acyl chain length below 22 are fluid at temperatures above 0 °C hydrophobic thickness rather than lipid fluidity seems to be the critical factor both in the absence and presence of cholesterol. That di-C14:0 supported higher activity than di-C14:1 could likewise be explained by the more favorable hydrophobic thickness of the former (Table 1). An increased bilayer thickness would also explain the activating effect of cholesterol on Na,K-ATPase reconstituted with either di-C14:0 PC or di-C18:0 PC (Figures 3 and 4) since inclusion of 40 mol % cholesterol, which is expected to increase d by about 20% (*34*) would increase the hydrophobic thickness to near optimal values for both. However, it cannot explain why di-C18:1 supported higher activity than di-C18:0 (Figure 4).

An integral protein incorporated into a lipid bilayer may influence the lipid domain formation due to the protein/lipid interaction. On the other hand, the lipid domain structure may change the tension on the protein, which may lead to a conformational change, e.g., by rearrangement or tilting of transmembrane segments. Hydrophobic matching of the lipid bilayer thickness and the hydrophobic core of the protein could, therefore, be an important driving force for the coupling between lipid structure and integral membrane proteins, hydrophobic coupling (*35*).

Hydrophobic matching is probably important in supporting optimal ATPase activity because the membrane hydrophobic thickness (d) must accommodate the hydrophobic core of the integral protein (δ) to avoid hydrophobic mismatch (d-δ). If the energetic penalty for exposing hydrophobic groups to water is much larger than the change in bending stress of the elastic bilayer, the change in bending energy will be proportional to the square of this hydrophobic mismatch (*36*). Therefore, changing the hydrophobic thickness of the membrane can influence the activation energy for certain protein reactions, for example, associated with conformational changes. Actually, as seen in Figure 8 this seems to be the case for cholesterol at all fixed phospholipid acyl chain length and for PC at varying acyl chain lengths.

Interestingly, the optimal phospholipid acyl chain length is demonstrated to be shorter for sarco(endo)plasmic reticulum Ca-ATPase (SERCA), which is found in cholesterol-poor membranes, than the Na,K-ATPase present in the cholesterol-rich plasma membrane. This could in part be due to the presence of the β-subunit in the latter. For Ca-ATPase the optimal catalytic activity is supported at an acyl chain length, $n_c = 18$ (22) comparable to a hydrophobic thickness of about 27 Å, which is close to the estimated hydrophobic stretch of the Ca-ATPase of about 22 Å deduced from the 3D crystal structure (*37*). The SERCA ATPase is presumably fine-tuned for optimal function in the cholesterol-poor membranes of the sarco(endo)plasmic reticulum.

Johansson et al. (*4*) previously found a broader distribution of PC acyl chain lengths, between 16 and 20, that supported optimal pig kidney Na,K-ATPase activity in a mixed-micelle system. The results are difficult to compare directly with the present ones since another enzyme source and a different methodology was used. However, qualitatively the results are in agreement indicating that the major factors determining ATPase activity are bilayer thickness and lipid acyl chain order rather than fluidity.

Acyl chain length dependent cholesterol activation has previously been demonstrated for reconstituted Ca-ATPase: With monounsaturated PC with acyl chain length of 14 there was a significant activation by cholesterol, whereas for di-C18:1 PC no effect of cholesterol was found (*38*). Also Na,K-ATPase activity measured in native membranes depleted of or enriched in cholesterol has been shown to increase with cholesterol content up to 40 mol % (*9*).

Cholesterol partitions into PC bilayers with its OH-group at the level of the ester carbonyl group of the phospholipid (*39*). This increases the order by restricting the flexing motion of adjacent phospholipid acyl chains, especially at the level of the rigid sterol backbone.

Since both cholesterol and unsaturated acyl chains increase bilayer thickness by increasing the acyl chain orientational order, the order parameter could in itself be important for inducing a higher Na,K-ATPase activity, for example, by affecting the tension on the Na,K-ATPase. This could lead to a change in the transmembrane orientation of α-helices (*40*), distribution of conformational states, or conformational mobility. Since the rate-limiting steps in the Na,K-ATPase reaction cycle (cf. Scheme 1) involves the major conformational transitions, the $E_2 \rightarrow E_1$ step (*31*) and the $E_1P \rightarrow E_2P$ transition, this could affect the turnover of the enzyme. This could explain the higher activity of Na,K-ATPase reconstituted with di-C18:1 than with di-C18:0 (Figure 4) even though the latter has a hydrophobic thickness closer to optimal than the latter. Sinensky et al. (*41*) reported a correlation between the increased lipid order parameter by increasing cholesterol and the decreasing Na,K-ATPase activity in CHO cells.

Specific interactions of cholesterol and the transmembrane segments of Na,K-ATPase may also be suggested from the present investigation, for example, by the results depicted in Figure 5. At the optimal phospholipid acyl chain length in the absence ($n_c = 22$) or presence of cholesterol ($n_c = 18$), a significant difference between the Na,K-ATPase activity and the Na-ATPase activity is found: With cholesterol and di-C18:1 PC a significant activation by K^+ is observed, i.e., the activity is increased by a factor of about 5 at 50 μM ATP and about 20 at 3 mM ATP. However, without cholesterol and using di-C22:1 PC almost no K^+-activation was observed and the Na-ATPase activity was found to be almost identical to the Na,K-ATPase activity. Since K^+-activation takes place by acceleration of the dephosphorylation step this could indicate specific effects of the lipids on the dephosphorylation rate during the Na-ATPase reaction. Further indications supporting this suggestion are described below. The question of lipid effects on the partial reactions will be addressed in a following paper (Cornelius, unpublished results). A specific interaction of cholesterol on cation binding has previously been demonstrated in red cells where the cytoplasmic Na^+-affinity was found to increase after membrane extraction of cholesterol (*42*). In contrast, external cation affinity was found to be insensitive to changes in membrane cholesterol (*8*). It is therefore considered unlikely that the observed absence of K^+-stimulation of Na,K-ATPase reconstituted with phospholipids in the absence of cholesterol is caused by a decreased affinity to extracellular K^+. Specific interaction of cholesterol on V_{max} of Na,K-ATPase has also previously been observed in red cells (*43*) and the lipid environment with a high cholesterol content has been suggested to explain the unusual temperature dependence of red cell Na,K-ATPase with a block of the $E_1P \rightarrow E_2P$ transition at 0 °C (*44*, *45*). In fact, cholesterol was later demonstrated to affect the steady-state E_1P/E_2P distribution in reconstituted Na,K-ATPase (*10*, *46*).

As seen from Table 2 the dephosphorylation rate constant is by far the lowest of the rate constants for the Na-ATPase reaction. Thus, for the Na-ATPase reaction the turnover number k_{cat} will depend in principle on a single elementary step, the dephosphorylation reaction. In the Na,K-ATPase reaction the maximum turnover at 20 °C for shark enzyme can be calculated as the ratio between the maximum hydrolytic activity (V_{max}) and the phosphorylation site concentration. In membrane-bound enzyme these values are found to be about 180 μmol·mg^{-1}·h^{-1} at 50 μM ATP and 2.5 nmol/mg, respectively, giving a $k_{cat} \approx 20$ s^{-1} at 25 °C. After reconstitution with di-C18:1 PC + 40 mol % cholesterol the values are found to 642 μmol·mg^{-1}·h^{-1} and 5.6 nmol/mg giving a $k_{cat} \approx 33$ s^{-1}. Any rate constant far in excess of these values is unlikely to be rate determining for the overall Na,K-ATPase reaction. Using the rate constants for the partial reactions given in Table 2 the rate of step 1 (~40 s^{-1} at 50 μM ATP) and step 3 (~100 s^{-1}) both approaches k_{cat} and are expected to contribute to rate determination. As seen from Figures 6 and 7 the temperature

sensitivity of the turnover number applies to the Arrhenius law and the Eyring plots appear linear for both the Na-ATPase and the Na,K-ATPase reactions indicating that whatever different the temperature sensitivity of the two rate determining rate constants in the latter case may be (cf. 47), it is too small to affect the linearity of the Eyring plot.

The temperature dependence of enzyme reconstituted with monounsaturated PC demonstrates that cholesterol increases the entropy of activation of both Na,K-ATPase activity and Na-activity (Figure 8). Furthermore, both reactions were also activated by saturated di-C14:0 PC as compared to the monounsaturated di-C14:1 PC. In the latter case, a similar high entropy of activation as with monounsaturated PC in the presence of cholesterol is found (Eyring plots of Figure 3 give $\Delta H^{\ddagger}$ of 84 ± 4 kJ/mol). Activation of turnover by cholesterol and saturated di-C14:0 PC thus both seem to be associated with an increased enthalpy of activation. Furthermore, both for monounsaturated PC in the presence of cholesterol and for saturated di-C14:0 PC activation of turnover was associated with positive values for $\Delta S^{\ddagger}$ (Eyring plots of Figure 3 give $\Delta S^{\ddagger}$ of 46 ± 2 J/K·mol). As positive $T\Delta S^{\ddagger}$ corresponds to a decrease in $\Delta G^{\ddagger}$ this means that entropy contribution to the activation energy decreased.

Positive values of entropy of activation have previously been found associated with charge neutralization during enzyme−substrate complex formation, with release of water molecules (*48*). Complex formation of ATP with myosin ATPase is, for example, associated with an entropy of activation of about 184 J/K·mol (*49*). A similar charge neutralization in connection with formation of the substrate−enzyme complex formation during the ATP binding reaction takes place for the Na,K-ATPase reaction (*50, 51*). Besides such charge neutralization, structural changes after ATP binding are probably responsible. Extensive structural changes are known to take place during turnover of the Na,K-ATPase reaction, as mentioned below, and configurational entropy could make a large positive contribution to $\Delta S^{\ddagger}$. This suggests that cholesterol affects some structural rearrangements occurring during enzyme turnover.

Both acyl chain saturation and cholesterol increases significantly the dipole potential of the bilayer (*52*). This would produce large differences in the electric field strength within the bilayer in the phospholipid headgroup region, which may modify any electrogenic steps along the reaction pathway. Both step 1 ($E_2 \rightarrow E_1$ reaction) and step 3 ($E_1P \rightarrow E_2P$) are associated with charge translocation (i.e., they are electrogenic steps), whereas step 4 ($E_2P \rightarrow E_2$) is electroneutral, at least at saturating extracellular K^+ (see ref *53*). It is possible, therefore, that electrostatic effects caused by the large dipole potential induced by cholesterol partition into the bilayer changed the polarity of the enzyme and that this is the reason for the change in entropy of activation. Alternatively, the increased tension on the protein induced by cholesterol and saturated PC could cause the Na,K-ATPase to be slightly more unfolded in the absence of cholesterol.

The free energy of activation is minimal at $n_c = 18$ in the presence of cholesterol, i.e., the same acyl chain length that gave maximum turnover. Likewise, without cholesterol the minimum $\Delta G^{\ddagger}$ value was observed at $n_c \geq 22$, the same that gave maximum turnover (Figure 8C). The minimal value of $\Delta G^{\ddagger} \sim 65$ kJ/mol for reconstituted Na,K-ATPase in the presence of cholesterol, where the maximum activity is equal to the one found in native membrane-bound enzyme, is similar to the one found in native membrane-bound enzyme where $\Delta G^{\ddagger} \sim 62$ kJ/mol (compare Figures 6 and 8), indicating identical pumping efficiency.

One could speculate which steps in the reaction Scheme 1 are the most likely to be affected by cholesterol and phospholipids. Since phospholipid acyl chain length and cholesterol change V_{max} they must as a minimum influence the rate-determining steps. During the Na,K-ATPase reaction, this is the $E_2 \rightarrow E_1ATP$ transition and the $E_1P \rightarrow E_2P$ reaction, whereas during the Na-ATPase reaction it is the dephosphorylation step, $E_2P \rightarrow E_2$. Furthermore, the presence of cholesterol has previously been shown to increase the steady-state EP-level of reconstituted Na,K-ATPase significantly (*10*). This demonstrates that apart from accelerating the rate determining reactions to increase V_{max} cholesterol must also change the balance between phosphorylation and dephosphorylation (steps 3 and 4 in Scheme 1). Thus, if k_4 is increased k_3 must be increased even further.

That the lipid effects could be on both the phosphorylation/dephosphorylation reaction (step 4 in Scheme 1) and on reactions involving E_1/E_2 conformational changes (steps 1 and 3 in Scheme 1) may seem surprising at first, since the phosphorylation domain is well separated from the transmembrane region as indicated by the crystal structure of the closely related Ca-ATPase (*37*). However, the mentioned reaction steps are all associated with major conformational changes and the marked effects of phospholipid chain length and cholesterol on the Na,K-ATPase reaction therefore presumably follow from changes in the conformational mobility of the Na,K-ATPase. The shark Na,K-ATPase is surrounded by about 60 phospholipid molecules in the membrane (*54, 55*), and their cooperative action could be sufficient to result in significant conformational effects. The crystal structure of the Ca-ATPase (*37*) together with TIDPC/16-labeling experiments using Na,K-ATPase from *Torpedo* (*56*) suggest that the lipid/protein contact is predominantly with M1, M3, M9, and M10, and to a lesser extent M2 and M7, whereas M4, M5, M6, and M8 are shielded at the interior of the complex. Furthermore, the Ca-ATPase model suggests that after the initial ATP-binding substantial movements of the nucleotide-binding (N), phosphorylation (P), and Actuator (A) domains take place (*37*). These movements must somehow be transmitted to the transmembrane region in association with the cation transport and the E_1/E_2 conformational change. Such lever-functions are believed to be associated with the A-domain, which is connected to the transmembrane domain through M1 and M3, and by the L67-loop connecting M6 and M7, which mediate interactions of the phosphorylation domain and the transmembrane domain (*37*). Therefore, at least M1, M3, and M7 have pivotal roles that could be susceptible to protein/lipid interactions.

To conclude, the present work of reconstitution presented here seems to exclude any specific importance of the very complex lipid composition of biological membranes on Na,K-ATPase reaction: A fluid membrane with PC chain length about 18 and cholesterol is sufficient to support optimal Na,K-ATPase activity. Also the free energy of

activation for Na,K-ATPase reconstituted with di-C18:1 PC + cholesterol that supports optimal turnover is similar to the one found for native, membrane-bound Na,K-ATPase indicating that the two membranes support equally effective pumps. On the other hand, large effects on the turnover of Na,K-ATPase are observed by changes in lipid acyl chain length, the degree of saturation, as well as of changes in the content of cholesterol. Important parameters necessary to explain these effects include (i) hydrophobic matching of the bilayer and the incorporated protein, indicating direct interaction with the transmembrane segments of the Na,K-ATPase, (ii) lipid order, and possibly (iii) bilayer field strength. The exact fluidity of the liquid-crystalline phase seems to be less important than lipid order and matching of the bilayer thickness with the hydrophobic portion of the protein. This is only true from the point of view of optimizing turnover rate; however, other factors such as protein regulation are most certainly very dependent on the specific lipid composition (*57*). Finally, cholesterol and phospholipid acyl chain length affect the thermodynamic properties of the enzyme. Together the data indicate that phospholipids and cholesterol interact directly with the transmembrane segments of the Na,K-ATPase providing hydrophobic matching with the hydrophobic core of the protein.

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