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

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ABSTRACT: The effects of phospholipid acyl chain length (n_c) and cholesterol on several partial reactions of Na,K-ATPase reconstituted into liposomes of defined lipid composition are described. This regards the E_1/E_2 equilibrium, the phosphoenzyme level, and the K^+-deocclusion reaction. In addition, the lipid effects on some steady-state properties were investigated. Finally, the effects of cholesterol on the temperature sensitivity of the phosphorylation and spontaneous dephosphorylation reactions were investigated. The fatty acid and cholesterol composition of the native Na,K-ATPase membrane preparation showed a remarkable similarity to the lipid composition known to support maximum hydrolytic capacity as determined from in vitro experiments. The main rate-determining step of the Na,K-ATPase reaction, the $E_2 \rightarrow E_1$ reaction, as well as several other partial reactions were accelerated by cholesterol. This regards the phosphorylation by ATP as well as the $E_1{\sim}P \rightarrow E_2{-}P$ reaction. Moreover, cholesterol shifted the E_1/E_2 equilibrium toward the E_1 conformation and increased the K^+-deocclusion rate. Finally, cholesterol significantly affected the temperature sensitivity of the spontaneous dephosphorylation reaction and the phosphorylation by ATP. The effects of cholesterol were not completely equivalent to those induced by increasing the phospholipid acyl chain length, indicating that the cholesterol effects are not entirely caused by increasing the hydrophobic bilayer thickness, which indicates an additional mechanism of action on the Na,K-ATPase.

The Na,K-ATPase[1] is a member of the P-type ion-transport ATPases that is present in animal plasma membranes and required for cellular homeostasis. The Na,K-ATPase couples the free energy of ATP hydrolysis to the establishment of the electrochemical gradients for Na^+ and K^+ across the plasma membrane. The catalytic coupling of ATP hydrolysis to ion transport includes the following steps:

$$E_2(K_2) + ATP + 3Na^+_{cyt} \leftrightarrow E_1ATP{\cdot}Na_3 + 2K^+_{cyt} \quad (1)$$

$$E_1ATP{\cdot}Na_3 \leftrightarrow E_1{\sim}P(Na_3) + ADP \quad (2)$$

$$E_1{\sim}P(Na_3) \leftrightarrow E_2{-}P + 3Na^+_{ext} \quad (3)$$

$$E_2{-}P + 2K^+_{ext} \leftrightarrow E_2(K_2) + Pi \quad (4)$$

The first steps are the ATP accelerated release of two occluded K^+ at the cytoplasmic face of the enzyme and subsequent binding of three cytoplasmic Na^+. This step is associated with a conformational change from the low Na^+/high K^+-affinity form (E_2) to the high Na^+/low K^+-affinity form (E_1) (eq 1). Then follows phosphorylation of the enzyme at D386 with the occlusion of Na^+ (eq 2). Via a conformational change from $E_1{\sim}P$ to $E_2{-}P$, the three Na^+ are released to the extracellular side (eq 3). Finally, two extracellular K^+ are bound, and dephosphorylation leads to the occlusion of the bound K^+ (eq 4). Two of the reactions, eqs 1 and 3, are associated with thermally driven transitions (i.e., conformational changes).

In a preceding investigation, the modulation of the steady-state catalytic activity of Na,K-ATPase by phospholipids and cholesterol was described, and hydrophobic mismatch was demonstrated to significantly influence catalytic activity (*1*). Thus, monounsaturated phospholipids with an acyl chain length, n_c, of 18 and inclusion of 40 mol % cholesterol were found to support optimal hydrolytic activity of reconstituted ATPase engaged in both the Na,K-ATPase and the Na-ATPase reaction (*1*). Moreover, the maximum acid-stable phosphoenzyme level, EP, measured in the presence of Na^+ and absence of K^+, as well as the activation by cytoplasmic Na^+, was found to be strongly dependent on the presence of cholesterol in the liposomes (*2*).

An important question to address is which steps of the reaction mechanism are modified by the protein/lipid interaction. The very complex lipid composition of the plasma membrane and formation of a specific lipid domain is probably very important in the regulation of Na,K-ATPase

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[1] Abbreviations: CHL, cholesterol; E_1, Na,K-ATPase form with high affinity towards ATP and Na^+; E_2, Na,K-ATPase form with high affinity towards K^+ and low affinity to ATP; EP, phosphoenzyme; $E_1{\sim}P$, ADP-sensitive phosphoenzyme; E_2-P, K^+-sensitive phosphoenzyme.

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(e.g., by protein kinases (*3*, *4*)), and this has been further emphasized by the recent finding that Na,K-ATPase is associated with caveolae/rafts (*5*) indicating an important function for cholesterol in Na,K-ATPase regulation and targeting (*6*). The question is, furthermore, if the very complex lipid composition of the plasma membrane has any bearing on the Na,K-ATPase activity per se by interaction with specific steps in the ATPase reaction mechanism. 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 effects of cholesterol on the partial reactions described above have been investigated by steady-state and time-resolved measurements using chemical quench and rapid mixing stopped-flow fluorescence measurements.

MATERIALS AND METHODS

Enzyme Preparation. Membrane-bound Na,K-ATPase (EC 3.6.1.37) from rectal glands of the shark Squalus acanthias was prepared as previously described (*8*). This involves isolation of well-defined membrane fragments by differential centrifugation following treatment of microsomes with low concentrations of DOC (~0.15%), which permeabilizes the microsomes and removes loosely attached proteins. The specific hydrolytic activity was 30−33 U/mg protein (37 °C) at standard conditions according to Ottolenghi (*9*). The protein content was determined according to Lowry et al. (*10*) using bovine serum albumin as standard.

Reconstitution. Functional reconstitution of shark Na,K-ATPase was achieved as previously described (*7*, *11*). Cosolubilization of shark Na,K-ATPase membranes and the lipids of choice at a protein/lipid weight ratio estimated to give a final ratio of either 1:20 or 1:5 was obtained using the nonionic detergent $C_{12}E_8$ (4 mg/mg protein). The higher protein content of the latter proteoliposomes was necessary for the RH421 fluorescence measurements. Liposomes containing reconstituted Na,K-ATPase spontaneously formed when the detergent was subsequently removed by addition of hydrophobic bio-beads. Unless otherwise stated, the proteoliposomes were produced in 30 mM NaCl, 200 mM sucrose, 2 mM $MgCl_2$, and 30 mM histidine, pH = 7.0.

The proteoliposomes were characterized morphologically and functionally before being used for quantitative experiments. This included determination of the pump density (protein content and liposome size distribution), symmetry of protein insertion (inside-out, i/o, right side out, r/o, and nonoriented, n/o), and maximum hydrolytic activity. This is an important point since several of these parameters depended on the lipid composition, as described in the preceding paper (*1*). In all experiments except for the fluorescence measurements, only i/o-oriented enzyme was probed, whereas r/o- and n/o-oriented enzyme was inactive because of either the shielding of the substrate site inside the liposomes (r/o) or to inhibition by ouabain, as described above.

The fraction of enzyme reconstituted as i/o was determined using functional tests as previously described in detail (*7*, *11*), modified using ^{32}[P]ATP (*12*) to increase sensitivity and accuracy. In this analysis, the fractional increase in hydrolytic activity after addition of K^+ to nigericin-treated proteoliposomes produced in the absence of K^+ is a measure of the i/o fraction relative to the total amount of enzyme, deduced from the optimal activity of proteoliposomes opened by the detergent $C_{12}E_8$. If the i/o fraction is very small, its estimation may become uncertain since in this case the determination of orientation relies on the accurate estimation of the difference between two nearly identical activities. This situation is especially pertinent in the case of enzyme reconstitution in the absence of cholesterol where both enzyme activity and the fraction of i/o-oriented enzyme decreases. Protein determination of reconstituted ATPase was performed according to the Peterson modification (*13*) of the Lowry method (*10*). Bovine serum albumin was run as standard.

Lipid Extraction and Analysis. The cholesterol content of the shark Na,K-ATPase preparation was measured by enzymatic assay (Sigma Diagnostics). The phospholipid content was determined by phosphorus assay as described by Mills et al. (*14*). Lipids were extracted by standard methods (*15*) using ultrapure grade chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene (0.01% w/v) as an antioxidant. Phospholipids were separated from other lipids by solid-phase extraction on Strata SI-2 silica cartidges (Phenomenex, Pennant Hills, NSW, Australia). Fatty acid analysis of the phospholipid fraction was conducted as described in detail elsewhere (*16*).

ATPase Activity. The rate of ATP hydrolysis of reconstituted i/o-oriented Na,K-ATPase was measured using [^{32}P]-ATP as described in ref *12* after preincubation of the proteoliposomes with Mg^{2+} (5 mM), P_i (1 mM), and ouabain (1 mM) to inhibit enzyme that was reconstituted in a nonoriented (n/o) fashion. r/o-oriented enzyme was inactive in the analysis since their substrate site is shielded inside the liposomes that are impermeable to ATP. The hydrolytic activity was determined in a test medium containing 50 μM ATP, 130 mM NaCl, 10 mM KCl, and 1 mM $MgCl_2$. 0.7 μM nigericin was included in the test medium to ensure rapid equilibration of K^+ across the proteoliposomes (*17*).

Phosphorylation of Na,K-ATPase from [γ-^{32}P]ATP (10 μM to 1 mM) was followed using rapid chemical quench as previously described (*17*) in a medium containing 4 mM $MgCl_2$, 30 mM NaCl, and 30 mM imidazole, pH 7.4.

The maximum steady-state phosphorylation level of reconstituted Na,K-ATPase was measured as the acid-stable phosphoenzyme in the presence of Na^+ and in the absence of K^+. At these conditions, the dephosphorylation step is slower by at least an order of magnitude as compared to the other steps in the reaction, and the main state populated is the phosphoenzyme.

At the experimental conditions used, only i/o-oriented enzyme is phosphorylated since r/o-oriented enzyme is devoid of ATP, and nonoriented enzyme is blocked by preincubation with ouabain in the presence of MgP_i as described in Materials and Methods. Thus, only the small fraction of i/o-oriented enzyme is phosphorylated, and to determine the phosphorylation level (nmol/mg of i/o-oriented protein) a precise measurement of this fraction is necessary for each experimental condition (*11*).

Dephosphorylation of Na,K-ATPase after ATP phosphorylation was measured in the absence (spontaneous dephosphorylation) and presence of K^+ by chasing with unlabeled ATP (1 mM) followed by addition of an acid-stopping

solution in the presence of 10 mM $MgCl_2$ as previously described (*17*).

RH421 Fluorescence Measurements. Time-resolved RH421 fluorescence associated with ATP phosphorylation of reconstituted Na,K-ATPase was measured using a rapid mixing stopped-flow spectroflurometer (SX.17MV, Applied Photophysics) as described in ref *18*. The proteoliposomes used had a high protein content (1:5 protein to lipid weight ratio). One syringe contained 400 μL of proteoliposomes (about 50 μg/mL Na,K-ATPase) and 200−400 nM RH421 in 1600 μL of 25 mM histidine buffer, and the second syringe contained a 2000 μL buffer of 10 mM HEPES plus 10 mM MES, or 30 mM imidazole, adjusted with *N*-methyl-D-glucamine to pH 7.5 in the presence of 4 mM $MgCl_2$ and various amounts of ATP. The RH421 concentration used is well below the inhibitory concentration of 1 μM. K^+-supported dephosphorylation was measured using proteoliposomes prephosphorylated by ATP in one syringe and 20 mM K^+ in the second syringe. The flow volume was 100−300 μL. The excitation wavelength was 546 nm (using a combined xenon/mercury lamp), and fluorescence was measured at emissions ≥630 nm using a cutoff filter. The dead time for the stopped-flow apparatus was about 1.5 ms.

Data Analysis. Mono- and double-exponential functions were used to fit the phosphorylation and dephosphorylation data. The goodness of the fits was quantified using an *F*-test and a 5% confidence level. If double-exponential time functions fitted the data significantly better than monoexponential equations, the observed rate constants (k_1, k_2) and the fractions for the rapid and slow phases (f_1, f_2) are given.

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. All solvents used in the lipid analysis were of ultrapure grade and were obtained from BDH. Analytical grade butylated hydroxytoluene was from Sigma.

RESULTS

Fatty Acid Composition. Table 1 shows the fatty acid composition of the shark rectal gland membrane preparation. The cholesterol/phospholipid ratio is high, amounting to 0.60 (mol/mol) reflecting, in part, the protein purification obtained by the DOC treatment. The unsaturation index of 210 (the number of double bonds per 100 fatty acid chains) is high with a high content of polyunsaturates, in particular n-3 and n-6 unsaturated lipids. As seen, the phospholipids are mainly composed of 16:0 (26%), 18:1 (15%), 20:4 (11%), and 22:6 (14%) acyl chains. The average phospholipid acyl chain length is 18.6 (Table 1) and 18.1, respectively, in two different preparations.

Rate-Determining Steps in the Na,K-ATPase Reaction Cycle. To regulate the maximum Na,K-ATPase turnover rate, the rate-limiting steps in the reaction cycle are likely to be affected. The Na,K-ATPase catalytic reaction is conventionally described by the Albers−Post scheme shown in a simplified version in Scheme 1. In the physiological Na,K-ATPase reaction at saturating substrate concentrations, the $E_2 \rightarrow E_1$ transition is the main rate-limiting step (*19*−*21*).

Table 1: Lipid Composition of Shark Rectal Na,K-ATPase Membrane Preparations

fatty acids	(% total)
14:0	0.32
16:0	25.74
17:0	0.64
18:0	6.13
19:0	0.26
20:0	0.25
14:1 n-7	0.25
16:1 n-7	1.81
17:1 n-7	1.03
18:1 n-9	15.48
18:1 n-7	6.35
18:2 n-6	1.77
18:3 n-6	0.47
18:3 n-3	0.54
20:1 n-9	3.09
20:2 n-6	0.31
20:3 n-9	0.12
20:3 n-6	0.16
20:4 n-6	11.11
20:3 n-3	0.14
20:5 n-3	4.88
22:3 n-3	2.06
22:4 n-6	0.86
22:5 n-3	1.74
22:6 n-3	14.48
% saturates	33.35
% mono-unsaturates	28.02
% polyunsaturates	38.64
% n-9	18.69
% n-7	9.45
% n-6	14.68
% n-3	23.84
unsaturation index	210
20 + 22C polyunsaturates	39.2
average chain length	18.6

Scheme 1

$$E_2(K_2) \underset{1}{\longleftrightarrow} E_1Na_3ATP \xrightarrow[2]{} E_1 \sim P \xrightarrow[3]{} E_2 - PK_2 \xrightarrow[4]{} E_2(K_2)$$

However, although it is the slowest step in the reaction cycle, step 3, the $E_1 \sim P \rightarrow E_2 - P$ transition has a rate constant only twice as large, at least at temperatures below 15 °C (*18*) and therefore also contributes to rate limitation of the enzyme turnover at physiological temperatures for the shark. At higher temperatures, however, this step becomes considerably faster and does not contribute to rate limitation of the pump cycle (*22*).

In the absence of K^+, the ATPase engages in the so-called Na^+/Na^+ exchange, where extracellular K^+ is replaced by Na^+. The associated ATP hydrolysis is termed the Na-ATPase activity. In the absence of K^+, the dephosphorylation rate decreases significantly and becomes by far the slowest step in the reaction (*23*) leading to an accumulation of enzyme species as EP.

E_1ATP → E_1∼P Reaction. As previously demonstrated, the $E_1ATP \rightarrow E_1 \sim P$ reaction (step 2 in reaction Scheme 1) is appreciably faster than the following $E_1 \sim P \rightarrow E_2 - P$ reaction, at least over the temperature range of 5−15 °C (*18*). Therefore, although both phosphoenzyme forms, $E_1 \sim P$ and $E_2 - P$, are acid-stable and detected by chemical-quench measurements, it is the rate of the initially formed $E_1 \sim P$ that is measured.

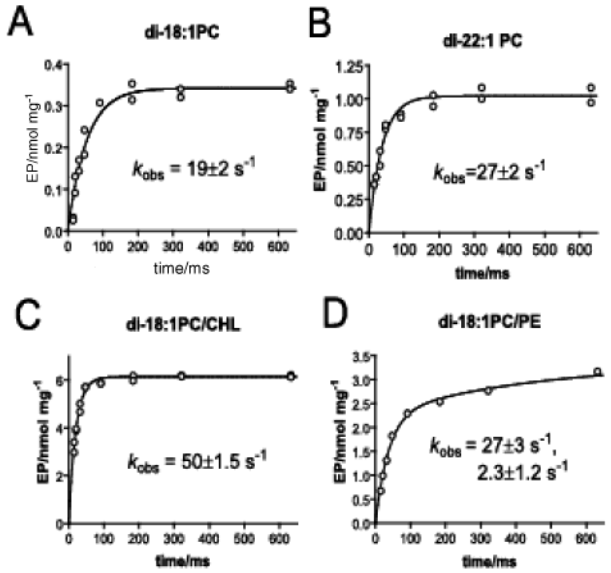

FIGURE 1: Phosphorylation of Na,K-ATPase reconstituted into liposomes with different lipid compositions. Enzyme phosphorylation by ATP was determined using chemical quenched-flow measurements at 10 °C. The conditions were ATP 25 μM, Na^+ 72.5 mM, sucrose 115 mM, Mg^{2+} 5 mM, Pi 0.5 mM, and imidazole 30 mM, pH 7.4. The proteoliposomes containing 30 mM Na^+ and 200 mM sucrose were preincubated with 15 mM Na^+, 230 mM sucrose, 5 mM Mg^{2+}, 1 mM Pi in imidazole buffer (pH 7.4) and 1 mM ouabain for 20 min at 23 °C to inhibit enzyme reconstituted with the extracellular side exposed (i.e., r/o and n/o enzyme, see Materials and Methods). The phosphorylation level of i/o-oriented Na,K-ATPase is given as nmol/mg. The curves are monoexponential fits to the data (except in panel D). The lipid composition of liposomes were A, di-18:1PC; B, di-22:1PC; C, di-18:1PC/cholesterol (60/40 mol/mol); and D, di-18:1 PC/phosphatidylethanolamine (85:15 mol/mol). The fitted rate constants were A, $k_{obs} = 18.7 \pm 1.9\ s^{-1}$; B, $k_{obs} = 26.8 \pm 1.6\ s^{-1}$; C, $k_{obs} = 49.9 \pm 1.5\ s^{-1}$. In D, double exponentials fit the data better. The fast phase ($f_1 = 0.67$) has a rate constant, $k_1 = 26.9 \pm 3.0\ s^{-1}$ and a slower phase ($f_1 = 0.33$) with rate constant $k_2 = 2.3 \pm 1.2\ s^{-1}$.

In Figure 1, the phosphorylation of i/o-oriented Na,K-ATPase by 25 μM ATP is measured at 10 °C after reconstitution with monounsaturated PC, either di-C18:1 PC, or di-C22:1 PC and compared with experiments where either 40 mol % cholesterol or 15 mol % PE is added in the reconstitution, conditions known to increase the hydrophobic thickness of the bilayer. For di-C18:1 PC and di-C22:1 PC, monoexponential time functions fitted the data satisfactorily with observed rate constants of 19 s^{-1}(18:1) and 27 s^{-1} (22:1), respectively (Figure 1A,B). In the presence of cholesterol, the rate constant increased by a factor of almost 3, whereas inclusion of PE created biphasic curves with a small phase with a 10 times lower rate constant (Figure 1D). For di-18:1 PC and di-18:1 PC/CHL, the apparent ATP affinity was about 3 μM. Addition of PE increased the apparent affinity for ATP to about 7 μM (not shown).

In the presence of cholesterol, the observed rate constant is comparable to the rate constant measured for membrane-bound Na,K-ATPase prior to solubilization and reconstitution, which was $77.3 \pm 3.4\ s^{-1}$ at 10 °C (*18*). Thus, the effects of cholesterol seem not to be associated solely with the increase in bilayer thickness since adjusting the phospholipid acyl chain length or inclusion of PE did not produce similar increase in the phosphorylation rate.

Another significant effect seen from Figure 1 is an increase in the steady-state phosphoenzyme level induced both by cholesterol and by PE. This effect will be considered in further details in the Discussion.

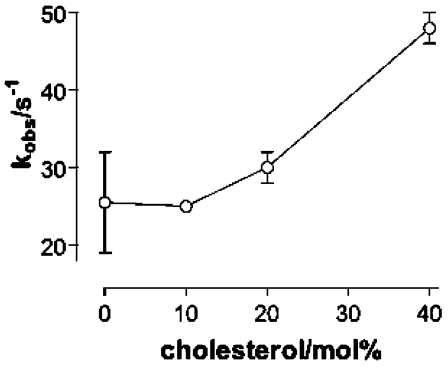

FIGURE 2: Rate of phosphorylation of Na,K-ATPase reconstituted with di-18:1PC and increasing cholesterol content measured by chemical quench-flow. The rate of ATP phosphorylation is determined by quench-flow measurements at 10 °C as described in the legend to Figure 1 and is depicted as a function of the cholesterol content of the proteoliposomes.

Figure 2 shows similar experiments produced with Na,K-ATPase reconstituted with di-18:1PC and increasing cholesterol content. As seen, cholesterol content above 10 mol % increases significantly the phosphorylation rate.

$E_2 \rightarrow E_1ATP$ and the $E_1{\sim}P \rightarrow E_2{-}P$ Reactions. As previously demonstrated, the phosphorylation from ATP can be measured in membrane-bound Na,K-ATPase by time-resolved fluorescence measurements using the potential sensitive styryl dye RH421, which specifically detects the formation of $E_2{-}P$ (*24*−*26*). Using proteoliposomes with a high protein/lipid ratio of 1:5, it is also possible to measure the RH421 fluorescence associated with ATP phosphorylation, as seen from Figure 3.

The two reactions, the $E_2 \rightarrow E_1ATP$ and the $E_1{\sim}P \rightarrow E_2{-}P$, can be monitored using the fluorescent dye RH421 by comparing enzyme phosphorylation from either the E_2 conformation or the E_1 conformation (*20*). This is achieved by comparing phosphorylation of enzyme preincubation in an E_2 stabilizing buffer like histidine in the absence of Na^+ and Mg^{2+} before addition of MgATP with enzyme preincubated with Na^+ and ATP (E_1 stabilizing conditions) before addition of Mg^{2+}. In the former condition, the rate of the reaction $E_2 \rightarrow E_1 \rightarrow E_1Na_3ATP \rightarrow E_1{\sim}P \rightarrow E_2{-}P$ is measured, which is rate limited by the initial $E_2 \rightarrow E_1$ conformational transition. In the latter condition, starting from the E_1 conformation, the formation of $E_2{-}P$ is rate limited by the phosphorylation reaction, $E_1 \rightarrow E_1{\sim}P$ (*27*), and only at low temperatures (<15 °C) will the following $E_1{\sim}P \rightarrow E_2{-}P$ reaction significantly contribute to rate determination (*18*).

In Figure 3, results are shown applying this approach using membrane-bound enzyme (panel A), enzyme reconstituted with di-18:1 PC in the absence of cholesterol (panel B), and enzyme reconstituted with the same phospholipid and 40 mol % cholesterol (panel C). For membrane-bound enzyme, the rate constant at 20 °C for phosphorylation decreased from about 150 to about 100 s^{-1} when the initial enzyme conformation was E_2 rather than E_1. Similar effects have previously been observed using pig or rabbit kidney enzyme (*20*). A similar decrease in the rate of phosphorylation is observed for reconstituted enzyme both in the absence and in the presence of cholesterol. Moreover, the presence of

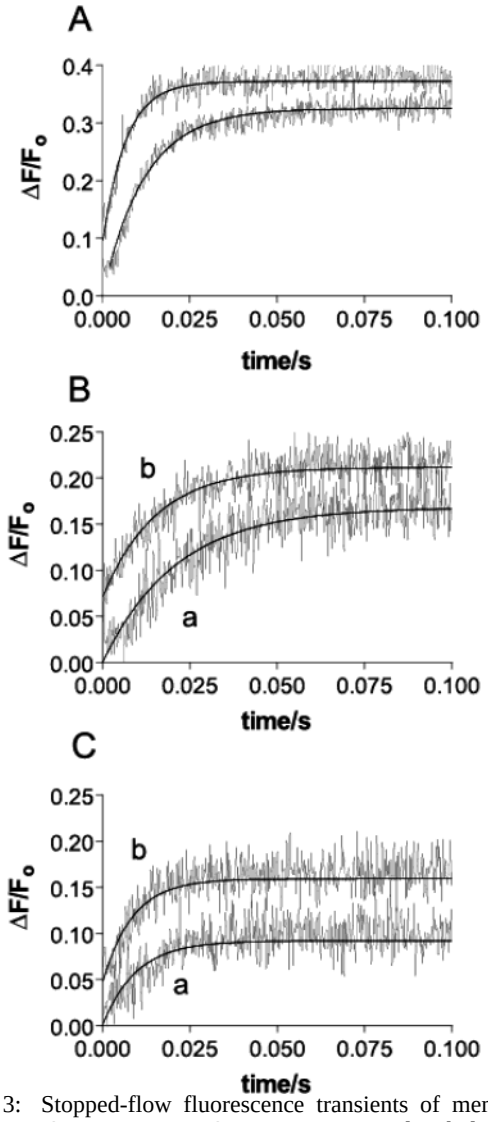

FIGURE 3: Stopped-flow fluorescence transients of membrane-bound Na,K-ATPase or Na,K-ATPase reconstituted with di-18:1PC with 40 mol % cholesterol or without cholesterol on mixing with ATP at 20 °C. In the responses labeled a, the enzyme in one syringe is initially stabilized in the E_1 conformation, whereas the responses labeled b are responses of enzyme initially stabilized in the E_2 conformation. Note that for clarity the a and b traces are displaced vertically. To stabilize the enzyme in the E_1 conditions, it was incubated in syringe one containing 50 mM NaCl, 2 mM ATP, 0.1 mM EDTA, and 30 mM histidine buffer, pH 7.4 together with the RH421 (200 nM, final). For the E_2 stabilizing conditions, NaCl and ATP were omitted. In cases where the enzyme was phosphorylated from the E_2 conformation, it was mixed with a second syringe containing 130 mM NaCl, 0.1 mM EDTA, 30 mM histidine buffer, pH 7.4, plus 2 mM ATP and 5 mM $MgCl_2$. In cases where the enzyme was phosphorylated from the E_1 conformation, the second syringe contained 130 mM NaCl, 0.1 mM EDTA, 30 mM histidine buffer, pH 7.4, plus 5 mM $MgCl_2$. The curves show the computer fit to the data using monoexponential time functions. For membrane-bound enzyme (A) the fitted rate constants were (a) $k_{obs} = 150 \pm 4.7\ s^{-1}$ and $\Delta F/F_o = 0.28$; (b) $k_{obs} = 82.6 \pm 1.5\ s^{-1}$ and $\Delta F/F_o = 0.32$. Panels B and C show results using enzyme reconstituted in liposomes containing 260 mM sucrose and 30 mM histidine, pH 7.4. For enzyme reconstituted without cholesterol (B) the fitted rate constants were (a) $65.5 \pm 2.7\ s^{-1}$ and (b) $47.6 \pm 2.3\ s^{-1}$. For enzyme reconstituted with 40 mol % cholesterol (C), the fitted rate constants were (a) $117 \pm 14\ s^{-1}$ and (b) $83.9 \pm 8.3\ s^{-1}$. In both cases, $\Delta F/F_o \sim 0.15$, when the data are adjusted to the amount of inside-out enzyme.

cholesterol increased the phosphorylation rates of enzyme preset to E_2 or E_1.

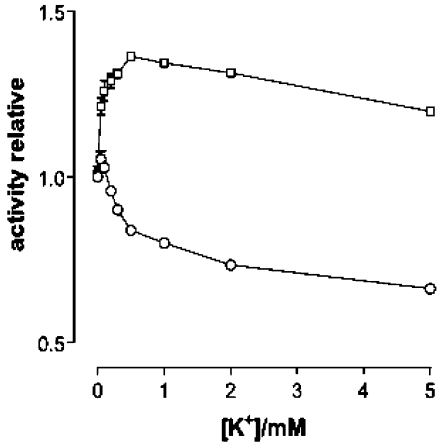

FIGURE 4: K^+ sensitivity of Na-ATPase activity of Na,K-ATPase reconstituted with di-18:1PC in the absence (upper curve) or presence of 40 mol % cholesterol (lower curve). Reconstituted Na,K-ATPase was preincubated with ouabain in the presence of MgPi to inhibit enzyme reconstituted as right-side out or as nonoriented as described in Materials and Methods. ATP hydrolysis of inside-out-oriented Na,K-ATPase was subsequently measured at 20 mM NaCl, 1 μM ATP, and varying K^+ concentrations between 0 and 5 mM at 20 °C. A total of 0.5 μg/mL nigericin was present to allow K^+ access to the extracellular side of the inside-out-oriented enzyme, as described in Materials and Methods. The data are expressed as fractions of the Na-ATPase activity measured in the absence of K^+. Values for a typical experiment are shown as the mean ± SEM of triplicate determinations.

It should be noted that since Mg^{2+} drives the enzyme into the E_1 conformation, it is not possible in these experiments to ensure complete inhibition of enzyme reconstituted as nonoriented by high-affinity ouabain binding (*28*) by pre-incubation in the presence of Mg^{2+} and Pi. Thus, in these experiments phosphorylation of enzyme, which is not properly imbedded into the lipid bilayer, cannot be avoided. However, this is not likely to affect the RH421 fluorescence, which senses events taken place in the membrane phase.

K^+-Deocclusion Reaction. The $E_2 \rightarrow E_1ATP$ reaction described above includes the K^+-deocclusion reaction that is accelerated by low-affinity ATP binding. At low ATP concentration, the deocclusion of K^+ may become rate limiting (*29*). Figure 4 shows K^+ activation at 1 μM ATP of Na,K-ATPase reconstituted in the absence and presence of 40 mol % cholesterol. In the absence of K^+, the Na-ATPase activity is measured. Addition of K^+ increases the hydrolytic activity indicating that the Na,K-ATPase activity is higher than the Na-ATPase activity. As K^+ increases, the Na,K-ATPase reaction may become increasingly rate limited by the K^+-deocclusion reaction. In the presence of cholesterol, this decrease in activity sets in at very low K^+ concentrations, whereas without cholesterol this decrease in hydrolytic activity is shifted to higher K^+ concentrations.

Spontaneous Dephosphorylation. Figure 5 depicts the spontaneous dephosphorylation at temperatures between 0 and 20 °C of phosphorylated Na,K-ATPase reconstituted with di-18:1 PC in the absence or presence of 40 mol % cholesterol. At 0 °C, the data could be fitted with monoexponential functions with rate constants of 0.133 ± 0.009 and $0.094 \pm 0.010\ s^{-1}$, demonstrating that cholesterol had no effect on the observed rate constant for spontaneous dephosphorylation at these conditions. However, this picture changed as the temperature was raised. If the same experiments were run at 20 °C, the rate constant for the spontaneous

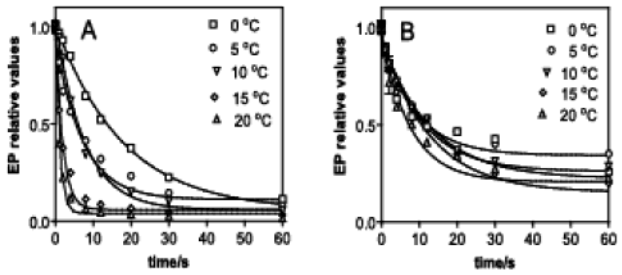

FIGURE 5: Temperature sensitivity of the dephosphorylation reaction of Na,K-ATPase reconstituted with di-18:1 PC with 40 mol % cholesterol (A) or without cholesterol (B). Reconstituted Na,K-ATPase was initially phosphorylated with ^{32}P-ATP after preincubation with ouabain in the presence of MgPi, as described in Materials and Methods, and the spontaneous dephosphorylation of inside-out-oriented Na,K-ATPase was followed with time by being chased with cold ATP and stopped by acid quenching. The data are given as relative to the initial phosphorylation level (EP) and fitted with monoexponential time functions shown as the curves in the figure. The fitted observed rate constants were used in the Eyring plot, shown in Figure 10.

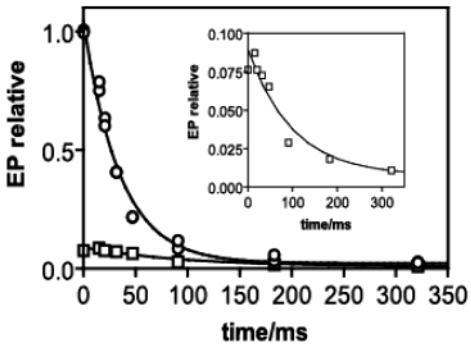

FIGURE 6: K^+-supported dephosphorylation at 10 °C of Na,K-ATPase reconstituted with di-18:1 PC with or without 40 mol % cholesterol. Reconstituted inside-out-oriented Na,K-ATPase was initially phosphorylated with ^{32}P-ATP as described in Materials and Methods, and the spontaneous dephosphorylation was followed with time by being chased with cold ATP in the presence of 20 mM KCl and stopped by acid quenching. Nigericin (0.5 μg/mL) was included in the chase solution to allow K^+ access to the extracellular side of the enzyme, inside the liposomes. The data are given as relative to the initial phosphorylation level (EP) for enzyme reconstituted enzyme in the presence of 40 mol % cholesterol and fitted with monoexponential time functions shown as the curves in the figure. The fitted observed rate constants were 28.7 $\pm$ 1.9 s^{-1} for enzyme reconstituted with cholesterol (○, upper curve) and 10.0 $\pm$ 2.3 s^{-1} for enzyme reconstituted without cholesterol (□, lower curve). The data for enzyme reconstituted in the absence of cholesterol is also given in the inset on an expanded time-scale.

dephosphorylation increased to 0.90 $\pm$ 0.05 s^{-1} in the presence of 40 mol % cholesterol (i.e., by a factor of 7), whereas in the absence of cholesterol it was 0.13 $\pm$ 0.02 s^{-1}, almost identical to the rate at 0 °C. Thus, the temperature sensitivity of the spontaneous dephosphorylation reaction is strongly dependent on the presence of cholesterol.

K^+-Supported Dephosphorylation. This was measured by including K^+ in the chase dephosphorylation solution. As shown in Figure 6, the spontaneous dephosphorylation at 10 °C is significantly accelerated by K^+ as expected. Both with and without 40 mol % cholesterol, the dephosphorylation rate constant was in the range of 10−30 s^{-1} as compared to the spontaneous dephosphorylation rate constant, which was about 0.1 s^{-1} (cf. Figure 5).

The phospholipid acyl chain length had a dramatic effect on the dephosphorylation rate. As demonstrated in Figure 7, in Na,K-ATPase reconstituted with C22:1 PC and 40 mol % cholesterol the dephosphorylation became biphasic with a slow component with a rate constant that decreased by a factor of more than 500 as compared to enzyme reconstituted with di-18:1 PC and 40 mol % cholesterol (cf. Figure 6). Furthermore, the dephosphorylation became essentially K^+ insensitive.

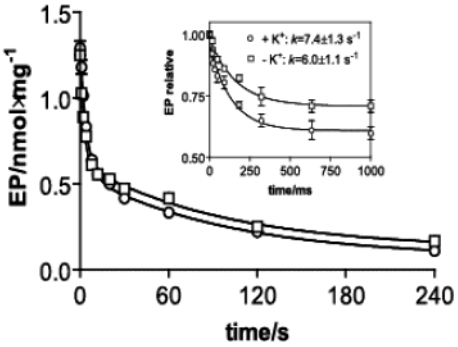

FIGURE 7: K^+-supported and spontaneous dephosphorylation at 10 °C of Na,K-ATPase reconstituted with di-22:1 PC with 40 mol % cholesterol. The incubations and measurements were as described in the legend to Figure 6. The dephosphorylation rates are identical with and without 20 mM K^+ in the ATP chase solution, and the time course is clearly biphasic on a time scale of seconds. The inset shows that in addition an initial fast dephosphorylation within the first second (rate constants 6.0 $\pm$ 1.1 and 7.4 $\pm$ 1.3 s^{-1} without and with 20 mM K^+, respectively) occurs. The rate constants for the slow phases of the dephosphorylations were 0.3 $\pm$ 0.03 and 0.01 $\pm$ 0.003 s^{-1} and identical with or without the presence of K^+ in the dephosphorylation reaction.

E_1/E_2 Equilibrium. Orthovanadate is a transition state homologue of inorganic phosphate that binds to the E_2 conformation of Na,K-ATPase (*30*) resulting in inhibition. Therefore, the Na,K-ATPase sensitivity to inhibition by vanadate can be used as a measure of the poise toward the E_2 conformation (*29*).

Figure 8 shows the vanadate sensitivity of Na,K-ATPase reconstituted in the absence or presence of 40 mol % cholesterol. The vanadate sensitivity of native membrane-bound shark enzyme (I_{50} about 0.5 μM, not shown) is comparable to kidney α1 isoform (*29*), whereas the reconstituted shark enzyme is about 1 order of magnitude less sensitive to vanadate inhibition. The experiment demonstrates that Na,K-ATPase is slightly more sensitive to vanadate inhibition in the absence of cholesterol (I_{50} = 2.6 μM) than in the presence (I_{50} = 10.0 μM) (difference significant with P = 0.001) that is, cholesterol poised the E_2/E_1 equilibrium toward E_1, but the effect is not dramatic . A similar effect of cholesterol has previously been found for the phosphoenzyme equilibrium where cholesterol poises the E_1P/E_2P equilibrium toward the E_1P (*2, 31*).

Activation Energie. The temperature sensitivity of the phosphorylation and dephosphorylation reactions was analyzed according to transition state theory where the rate constant, k, for an elementary chemical reaction follows the equation

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

where k_B is the Boltzmann constant, h Planck's constant, R the gas constant, T the temperature, $\Delta S^{\ddagger}$ the entropy of activation, and $\Delta H^{\ddagger}$ the enthalpy of activation. The values

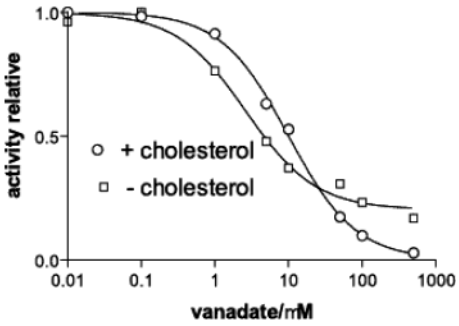

FIGURE 8: Vanadate sensitivity of Na,K-ATPase reconstituted with di-18:1 PC with or without 40 mol % cholesterol. ATP hydrolytic activity was measured at 100 mM NaCl, 10 mM KCl, 50 mM sucrose, 1 mM MgI_2, 10 μM ATP, 0.5 μg/mL nigericin, and 30 mM histidine pH 7.0. The proteoliposomes were preincubated with ouabain in the presence of MgPi to inhibit enzyme reconstituted with orientations other than inside-out, as described in Materials and Methods. Data are presented as fractions of the activity measured in the absence of vanadate. Means ± SEM of triplicate determinations are shown for a typical experiment. Symbols are ○, enzyme reconstituted with di-18:1PC/CHL (60:40 mol/mol) and □, enzyme reconstituted with di-18:1PC without cholesterol. The curves are fit using a one site competition model. In the fitted constants, I_{50}, the half-maximal inhibitor concentrations equal 10 ± 1.1 μM in the presence of cholesterol and 2.6 ± 1.2 μM in the absence of cholesterol (significant difference with $P = 0.0001$).

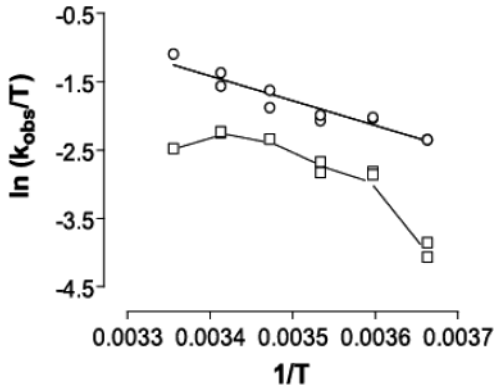

FIGURE 9: Eyring plot showing the temperature sensitivity of ATP phosphorylation for Na,K-ATPase reconstituted with di-18:1PC in the presence or absence of 40 mol % cholesterol. The observed rate constants measured at various temperatures for the ATP phosphorylation of reconstituted inside-out-oriented enzyme in the presence of cholesterol (○) or without cholesterol (□) as depicted in Figure 1 are shown in an Eyring plot. From the straight-line relationship for enzyme reconstituted in the presence of cholesterol, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be determined from the slope ($-\Delta H^{\ddagger}/R$) and intercept ($\Delta S^{\ddagger}/R + 23.76$) as $\Delta H^{\ddagger} = 30 \pm 3$ kJ/mol and $\Delta S^{\ddagger} = -107 \pm 13$ J/mol K.

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 $\ln(k_{obs}/T)$ axis of the linear plot of $\ln(k_{obs}/T)$ 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.

The Eyring plot for phosphorylation of Na,K-ATPase reconstituted with di-C18:1 PC in the presence and absence of cholesterol is shown in Figure 9. The curvature of the Eyring plots is pronounced in the absence of cholesterol, whereas in the presence of cholesterol the plot appears more linear. In the presence of Na^+, the phosphorylation reaction includes the reactions $E_1 \leftrightarrow E_1ATP \rightarrow E_1{\sim}P \leftrightarrow E_2{-}P \rightarrow E_1$. The phosphorylation reaction is measured in the absence of K^+, which means that the dephosphorylation rate constant

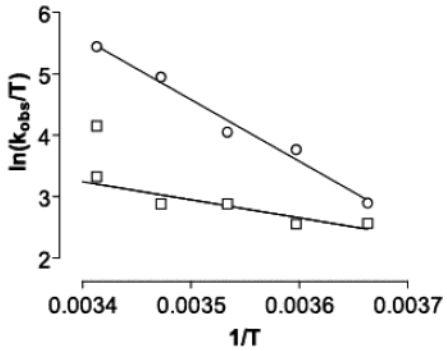

FIGURE 10: Eyring plot showing the temperature sensitivity of the spontaneous dephosphorylation for Na,K-ATPase reconstituted with di-18:1PC in the presence or absence of 40 mol % cholesterol. The observed rate constants for the spontaneous dephosphorylation of reconstituted inside-out-oriented enzyme in the presence of cholesterol (○) or without cholesterol (□) measured at various temperatures as depicted in Figure 5 are shown in an Eyring plot. From the slope ($-\Delta H^{\ddagger}/R$) and intercept ($\Delta S^{\ddagger}/R + 23.76$) of the straight-line relationships, the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be determined as $\Delta H^{\ddagger} = 94 \pm 15$ kJ/mol and $\Delta S^{\ddagger} = 167 \pm 24$ J/mol K in the presence of cholesterol and $\Delta H^{\ddagger} = 12 \pm 9$ kJ/mol and $\Delta S^{\ddagger} = -129 \pm 63$ J/mol K in the absence of cholesterol. Note the significantly smaller enthalpy of activation for enzyme reconstituted in the absence of cholesterol and the negative entropy of activation.

is very small as compared to the other rate constants around the reaction cycle. The formation of $EP = E_1{\sim}P + E_2{-}P$ will, however, depend on a minimum of the remaining four involved rate constants. Thus, the curved Eyring plots in the absence of cholesterol probably reflect the different temperature sensitivity of the involved rate constants. In the presence of cholesterol, the thermodynamic parameters calculated from the apparent straight line were $\Delta H^{\ddagger} = 30$ kJ/mol and $\Delta S^{\ddagger} = -107$ J/K mol. Thus, the entropy of activation is contributing to the free energy change of activation following phosphorylation, which can be calculated to $\Delta G^{\ddagger} \sim 62$ kJ/mol at 25 °C.

In contrast to the phosphorylation reaction, the dephosphorylation involves only a single rate constant, k_5, in Scheme 1 and should apply strictly to the Arrhenius law. The temperature sensitivity of the spontaneous dephosphorylation reaction of Na,K-ATPase reconstituted with di-C18:1 PC in the absence of cholesterol is much lower than in the presence of 40 mol % cholesterol (Figure 5).

This difference is reflected in the lower slope of the Eyring plot of enzyme reconstituted in the absence of cholesterol (Figure 10), which is apparently linear both in the absence and in the presence of cholesterol. From the slope, the enthalpy of activation can be calculated to $\Delta H^{\ddagger} = 94$ kJ/mol in the presence of cholesterol, whereas in the absence of cholesterol a significantly lower value of 12 ± 9 kJ/mol was obtained. The entropy of activation was $\Delta S^{\ddagger} = 167 \pm 24$ J/mol K and -129 ± 63 J/K mol, respectively. Thus, the free energy of activation at 25 °C was similar $\Delta G^{\ddagger} \sim 44 \pm 7$ and 50 ± 41 kJ/mol, respectively but mainly enthalpic in the presence of cholesterol, whereas in the absence of cholesterol it was mainly entropic.

DISCUSSION

It has previously been demonstrated that a specific phospholipid acyl chain length and the presence of cholesterol (*1*, *2*, *32*, *33*) is essential to support optimal hydrolytic

activity of Na,K-ATPase. Thus, monounsaturated acyl chain lengths, $n_c = 22$ in the absence and 18 in the presence of 40 mol % cholesterol is optimal for hydrolytic activity of reconstituted Na,K-ATPase. Furthermore, cholesterol significantly increased the maximum steady-state phosphoenzyme level and decreased the apparent cytoplasmic Na^+ affinity (*2*).

As seen from Table 1, the high cholesterol content of the shark membrane preparation and the average phospholipid acyl chain length of about 18 is remarkably close to the conditions shown to be optimal for supporting maximal turnover of the Na,K-ATPase (*1*). Previous comparisons of fatty acids from microsomal membranes of mammals and ectotherms reveal greater levels of polyunsaturation in mammalian tissues, with the brain having a high content of n-3 fatty acids, while the kidney has a high n-6 content (*34*). The brain is generally the most polyunsaturated organ in most species, and the rectal gland has a composition (unsaturation index and chain length) that is similar to the rat brain. Despite this high level of polyunsaturation when compared to mammals and other ectotherms, the n-3 content, unsaturation index, % polyunsaturation, and chain length are lower than may have been expected in a marine species that lives at low temperatures, as cold-water fish tend to have very high levels of polyunsaturation, and in particular, n-3 fatty acids (*35*). The high levels of urea in the spiny dogfish, however, may have affected the membranes of the rectal gland (*36*), resulting in a fatty acid composition that when coupled with the high level of cholesterol may be acting to give the membrane a conformation (bilayer thickness, fluidity, etc.) that is optimal for function.

The molecular mechanisms of these protein/lipid interactions are largely unknown but probably involve hydrophobic matching between the hydrophobic core of the integral protein and the bilayer to optimize the conformational mobility of the protein, as well as more specific protein/cholesterol interactions (*1*). The latter is further supported by the present investigation that specific steps in the Na,K-ATPase reaction cycle are affected by the bilayer composition.

The increase in a steady-state V_{max} for the Na,K-ATPase reaction at saturating substrate concentrations, as well as in the maximum phosphoenzyme level by cholesterol (Figure 1), must mean that cholesterol affects some of the rate-determining steps during Na,K-ATPase turnover. Regarding Na,K-ATPase, these includes the $E_2(K_2) \rightarrow E_1ATP(Na_3)$ reaction that was recently demonstrated to be the main rate-determining step at physiological conditions (*20*, *21*). Moreover, at temperatures below 15 °C, the $E_1{\sim}P(Na_3) \rightarrow E_2{-}P$ reaction (step 3 in the reaction Scheme 1) may also contribute significantly in rate limiting the overall reaction cycle (*18*), whereas at higher temperatures step 2, the $E_1 \rightarrow E_1{\sim}P$ reaction, makes a more important contribution to rate determination of the reaction cycle (*20*, *27)*. In contrast to phosphorylation, the dephosphorylation step is fast in the presence of K^+ and only during Na-ATPase conditions; in the absence of K^+, this reaction becomes the main rate-determining step.

As for kidney Na,K-ATPase, the present investigation demonstrates that the $E_2 \rightarrow E_1$ reaction rate limits the following phosphorylation reaction in both membrane-bound and reconstituted shark enzyme since phosphorylation starting with enzyme in the E_1 conformation is about twice as fast as phosphorylation starting from the E_2 conformation (Figure 3). Furthermore, the phosphorylation reaction of enzyme initially in the E_2 conformation is accelerated by cholesterol by a factor of about 2 (Figure 3B,C) demonstrating the cholesterol acceleration of the $E_2 \rightarrow E_1$ reaction. This is also the case when the phosphorylation reaction is started from enzyme initially in the E_1 conformation demonstrating that the $E_1 \rightarrow E_2{-}P$ reaction is also accelerated by cholesterol by a factor of 2−3. Nevertheless, the combined effects of cholesterol on these two rate-determining steps may not be adequate to fully explain quantitatively the increase in V_{max}, which can be increased by cholesterol by a factor of 10 (*2*). However, the measurement of the very low specific hydrolytic activity in the absence of cholesterol relies on a very precise determination of the enzyme orientation after reconstitution (i.e., on the proper estimation of the fraction of total enzyme reconstituted in the inside-out orientation) from functional tests (see Materials and Methods). In the absence of cholesterol, where the activity is small, this calculation becomes increasingly uncertain. Furthermore, the increase in the $E_2 \rightarrow E_1$ and $E_1 \rightarrow E_2{-}P$ forward reactions by cholesterol shown in Figure 3 may be a lower limit for the acceleration of enzyme turnover since the backward reactions may be affected (decreased) as well.

As seen from Figure 1, and as previously demonstrated (*2*), the population of the phosphorylated states, $E_1{\sim}P + E_2{-}P$, measured at steady-state in the absence of K^+ was strongly dependent on the cholesterol content of the proteoliposomes increasing by a factor of about 10 at 10 °C as the cholesterol content increases from 0 to 40 mol %. As for the estimation of V_{max}, the measurements of EP in the absence of cholesterol are subject to the same uncertainties of determination of enzyme orientation after reconstitution, described above. The rate of phosphorylation increases by a factor of about 3 by increasing the cholesterol content from 0 to 40 mol % under the same conditions and at 10 °C, whereas the dephosphorylation in the absence of K^+ (spontaneous dephosphorylation) is almost constant at this temperature as the cholesterol increases from 0 to 40 mol %. Therefore, a change in the phosphorylation/dephosphorylation forward reactions cannot alone fully account for the increase in maximum EP as cholesterol increases, suggesting that additional steps in the reaction mechanism are affected. One such step could be the K^+-deocclusion reaction: $E_2(K_2) \rightarrow E_1 + K^+$. As seen from Figure 4, cholesterol influences the K^+ activation at conditions where this step is rate determining. It is also conceivable, for example, that the backward reactions in the phosphorylation/dephosphorylation reactions could be affected by cholesterol.

Another possibility is that the balance between acid-stable and acid-labile EP is changed by cholesterol, a balance governed by the reaction $E{\cdot}P \rightarrow E{-}P$ where $E{-}P$ is the acid-stable pool of phosphoenzymes ($E_1{\sim}P + E_2{-}P$). We have previously shown that a significant portion of the phosphoenzyme must be acid-labile in shark Na,K-ATPase preparations (*24*). It is conceivable, therefore, that this reaction is influenced by the cholesterol content, so that low cholesterol poises the equilibrium toward the acid-labile form.

Forbush (*37*) has previously investigated the $E{\cdot}P \rightarrow E{-}P$ reaction comparing the acid-stop assay using TCA with a neutral assay employing cold methanol as the initial protein

precipitation step. Using a similar approach comparing acid and neutral precipitation methods, we were unable, however, to show any change in the low EP levels in Na,K-ATPase reconstituted without cholesterol.

We have previously shown that in enzyme reconstituted with 22:1PC, the Na-ATPase and Na,K-ATPase activity becomes similar (i.e., K^+ does not activate hydrolytic activity as is the case for native membrane-bound enzyme and enzyme reconstituted with a shorter acyl chain PC (*1*)). As seen from Figure 7, the reason for this can now be assigned to a specific effect of lipid acyl chain length on the dephosphorylation reaction. Thus, the dephosphorylation of enzyme reconstituted with 22:1PC, which is the rate-limiting step for Na-ATPase activity, is no longer accelerated by K^+ indicating that proper matching of the hydrophobic length of the bilayer is essential for the conformational mobility of the enzyme associated with the K^+ occlusion reaction associated with dephosphorylation: $E_2PK^+_2 \rightarrow E_2(K^+_2) + Pi$.

In conclusion, the lipid environment, and especially the cholesterol content, affects the hydrolytic activity of Na,K-ATPase by affecting several of the reaction steps in the overall reaction mechanism of the Na,K-ATPase and not just a few rate-determining steps. Indeed, both rate-limiting steps, the $E_2 \rightarrow E_1$ and the $E_1 \rightarrow E_2-P$ reactions, are accelerated by cholesterol. The $E_2 \rightarrow E_1$ reaction includes binding of the cytoplasmic ligands ATP and Na^+, as well as deocclusion of K^+, reactions which all seem to be affected by the lipid environment. The effect on the apparent affinities of ATP and Na^+ may both be caused by the lipid effect on the E_1/E_2 equilibrium. The detailed molecular mechanisms by which such effects are achieved are still largely unknown, but hydrophobic matching between the bilayer and the integral protein seems important. That this is not the only effect in play is apparent from the fact that a change in bilayer hydrophobic thickness induced by either increasing the phospholipid acyl chain length or by inclusion of cholesterol does not always affect the enzyme equivalently, as seen, for example, in the phosphorylation reactions depicted in Figure 1. Thus, effects on the conformational mobility of the Na,K-ATPase by the lipids seem to be important as indicated by the effects on the activation energy of the reactions accompanying the conformational changes associated with the phosphorylation/dephosphorylation reactions. Such effects could be direct or induced by phospholipid and cholesterol effects on the intramembrane charge distribution (cf. *22*) or result from certain lipid combinations that stabilize oligomeric interactions of the Na,K-ATPase.

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