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Fluorescence Spectroscopic Studies of Pressure Effects on Na^+,K^+-ATPase Reconstituted into Phospholipid Bilayers and Model Raft Mixtures†

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ABSTRACT: To contribute to the understanding of membrane protein function upon application of pressure as relevant for understanding, for example, the physiology of deep sea organisms or for baroenzymological biotechnical processes, we investigated the influence of hydrostatic pressure on the activity of Na^+,K^+-ATPase enriched in the plasma membrane from rabbit kidney outer medulla using a kinetic assay that couples ATP hydrolysis to NADH oxidation. The data show that the activity of Na^+,K^+-ATPase is reversibly inhibited by pressures below 2 kbar. At higher pressures, the enzyme is irreversibly inactivated. To be able to explore the effect of the lipid matrix on enzyme activity, the enzyme was also reconstituted into various lipid bilayer systems of different chain length, conformation, phase state, and heterogeneity including model raft mixtures. To yield additional information on the conformation and phase state of the lipid bilayer systems, generalized polarization values by the Laurdan fluorescence technique were determined as well. Incorporation of the enzyme leads to a significant increase of the lipid chain order. Generally, similar to the enzyme activity in the natural plasma membrane, high hydrostatic pressures lead to a decline of the activity of the enzyme reconstituted into the various lipid bilayer systems, and in most cases, a multi-phasic behavior is observed. Interestingly, in the low-pressure region, around 100 bar, a significant increase of activity is observed for the enzyme reconstituted into DMPC and DOPC bilayers. Above 100−200 bar, this activity enhancement is followed by a steep decrease of activity up to about 800 bar, where a more or less broad plateau value is reached. The enzyme activity decreases to zero around 2 kbar for all reconstituted systems measured. A different scenario is observed for the effect of pressure on the enzyme activity in the model raft mixture. The coexistence of liquid-ordered and liquid-disordered domains with the possibility of lipid sorting in this lipid mixture leads to a reduced pressure sensitivity in the medium-pressure range. The decrease of ATPase activity may be induced by an increasing hydrophobic mismatch, leading to a decrease of the conformational dynamics of the protein and eventually subunit rearrangement. High pressures, above about 2.2 kbar, irreversibly change protein conformation, probably because of the dissociation and partial unfolding of the subunits.

Cell membranes constitute one of the fundamental structural and functional elements of living organisms (*1*, *2*). The plasma membrane that encloses every cell defines the cell's boundary, maintains the essential difference between its contents and the environment, serves as a highly selective filter, which controls the movement of substances into and out of the cell, and regulates the composition of the fluid within individual cells. An important example of a membrane protein is the pump that regulates the concentration of sodium and potassium ions in the cell, the Na^+,K^+-ATPase (EC 3.6.1.37), present in the plasma membrane of nearly all cells in higher organisms. Na^+,K^+-ATPase is an integral membrane protein, which is responsible for the active transport of sodium and potassium ions through the plasma membrane coupled to adenosine 5′-triphosphate (ATP[1]) hydrolysis (*3*−*7*). It is the physiological key to the existence of an asymmetrical distribution of Na^+ and K^+ across the biological membranes of eukaryotic animal cells and is responsible for a variety of important biological functions, such as the

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; Chol, cholesterol; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Laurdan, 6-dodecanoyl-2-dimethylamino-napthalene; PK/L-LDH, pyruvate-kinase/L-lactate dehydrogenase; ATP, adenosine 5′-triphosphate; NADH, *β*-nicotinamide-adenine dinucleotide; PEP, phosphoenolpyruvic acid; *GP*, generalized polarization; FRET, fluorescence energy transfer.

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control of cell volume, the generation of resting potential in excitable cells, the secondary active transport of vital substances (glucose, amino acids, etc.), and heat production (*8*−*12*). During its catalytic cycle, Na^+,K^+-ATPase uses the energy of ATP hydrolysis to undergo conformational transitions between two main conformational states, E_1 and E_2, and to attain selective binding, occlusion, and release of Na^+ and K^+. Na^+,K^+-ATPase is classified as a P-type ATPase because the ATP hydrolysis reaction, in the presence of Na^+, involves the formation of an acid anhydride between phosphate and the Asp 369 amino acid residue located in the catalytic ATP site (*13*).

Na^+,K^+-ATPase is a heterodimer consisting of three subunits: an α-subunit, with a molecular mass of about 110 kDa, a highly glycosylated β-subunit, with a molecular mass of about 50 kDa, and a γ-subunit with an apparent molecular mass of about 12 kDa. The α-subunit contains the catalytic center required for ATP hydrolysis. It contains the ATP binding and phosphorylation domains as well as the essential amino acids for the binding of sodium, potassium, and some inhibitors. The β-subunit has a structural function and is important for the membrane insertion of the enzyme (*3*, *4*, *7*, *14*). A small hydrophobic protein, the γ-subunit (FXYD2), has been shown to associate with the α-subunit of Na^+,K^+-ATPase in a tissue-specific manner, although its function is still unclear. The γ-subunit is not essential for the Na^+,K^+-ATPase activity, but modulates the affinity of the enzyme for ATP and K^+ (*8*, *9*). Recent data suggest that this subunit is also able to enhance the maximal rate of enzyme catalysis (*15*). In the plasma membrane, the α- and β-subunits build functional oligomers of the $(\alpha\beta)_2$ type or oligomers with higher stoichiometries (*16*, *17*). In terms of gross structural features, approximately two-thirds of the total enzyme protrudes from the lipid bilayer and is in contact with the aqueous environment, whereas the remaining transmembrane portion is surrounded by the lipid bilayer. Na^+,K^+-ATPase is specifically inhibited by cardiac glycosides such as ouabain that preferentially bind to the extracellular loop domains (H1−H2, H3−H4, H7−H8) when the enzyme is in the phosphorylated E2-conformation (*18*, *19*).

All membrane proteins interact with lipids, specifically phospholipids and cholesterol. Most of the head groups of common phospholipids contain charged or highly polar groups. The acyl chains of mammalian phospholipids vary from 14 to 22 carbon atoms in length. Saturated phospholipids often exhibit two thermotropic lamellar phase transitions, a gel-to-gel ($L_{\beta'}/P_{\beta'}$) pre-transition and a gel-to-liquid-crystalline ($P_{\beta'}/L_\alpha$) main transition at a higher temperature T_m (*20*). In the fluid-like L_α phase, the hydrocarbon chains of the lipid bilayers are conformationally disordered, whereas in the gel phases, the chains are more extended and ordered. In addition to these thermotropic phase transitions, high hydrostatic pressure-induced phase transformations have also been observed (*21*, *22*). Phospholipid bilayer systems are important model systems for biological membranes and have been extensively studied with regard to structure, phase transitions, transport, and elasticity properties as well as with regard to the interaction with peptides, proteins, and other biomolecules.

In the past few years, there has also been a significant interest in the existence, in cell membranes, of lipid microdomains called lipid rafts, which may play an important role in many biological processes. Proteins may be selectively included or excluded from these micro-domains, which are postulated to have, for example, important roles in membrane signal transduction (*23*, *24*). They may also serve as portals of entry for various pathogens, including viruses, bacteria, and their toxins. The ordered raft domains were found to be highly enriched in sphingolipids and cholesterol. The presence of the long and saturated acyl chains in sphingolipids allows cholesterol to become tightly intercalated with such lipids, resulting in the organization of liquid-ordered (l_o) phases. By contrast, unsaturated phospholipids are loosely packed and form disordered domains (usually denoted as liquid-crystalline or liquid-disordered (l_d)). The rafts in cells appear to be heterogeneous, both in terms of their protein and their lipid content and can be localized in different regions of the cell. Suitable model systems for studying the biophysical properties of lipid rafts are lipid vesicles composed of three-component lipid mixtures such as POPC/SM/cholesterol, which exhibit a rich phase diagram, including raft-like liquid-ordered/liquid-disordered phase (domain) coexistence regions (*25*−*28*).

Although investigations on Na^+, K^+-ATPase have been continuing for more than 40 years, many aspects of this ion pump are still largely unknown (*29*). An important goal in studying membrane transporters is to characterize various aspects of their transport function. Initial studies were often carried out in intact cells, but further work has also been directed toward studying the functional properties of the purified protein. Reconstituted systems have proven to be extremely useful in the study of membrane transporters. They are also excellent systems for analyzing other properties, such as the influence of lipid composition, lipid−protein interactions, and topological asymmetry of the enzymatic and transport activities. Moreover, they allow a detailed spectroscopic characterization of the purified transporter using, for example, techniques such as fluorescence spectroscopy (*30*).

Temperature and hydrostatic pressure are well-known parameters, also often used in studies of biomolecular systems. Mostly, temperature has been used to study the thermodynamic, structural, and dynamic properties of membranes. A change in temperature of a system leads to changes of the thermal energy and density at the same time, whereas pressure-dependent studies at constant temperature introduce only changes in density and the intermolecular separations of the system, thus providing additional information about the energetics and phase behavior of the system without disturbing thermally activated processes (*27*, *28*, *31*). In physicochemical and biophysical studies, the interest in pressure as a variable to also study protein unfolding and refolding has been growing over the last few years (*27*, *28*, *32*, *33*). Very little is known about pressure effects on membrane proteins. However, some groups have described very interesting pressure-induced effects on enzymatic catalysis by membrane proteins (*34*−*40*). Generally, pressure affects chemical equilibria and reaction rates. For example, the activation volume, $\Delta V^{\neq}$, of a reaction is given by the pressure dependence of the rate of reaction, k. Therefore, $\Delta V^{\neq} = -RT(\partial \ln k/\partial p)_T$. High hydrostatic pressure has also been exploited in diverse areas of biotechnology, including the ability to modify the catalytic specificity of enzymes (*41*). Reasons for high hydrostatic pressure-induced changes in

the rate of enzyme-catalyzed reactions may be classified into three main groups: (i) changes in the structure of the enzyme, (ii) changes in the reaction mechanism or changes in the overall rate by affecting a particular rate-determining step, and (iii) the effect of pressure on the function of membrane proteins, which might also be due to an increase in lipid-packing density or a change in phase state, which might lead to a change in the conformation and dynamics of the embedded protein.

In this study, using fluorescence spectroscopic techniques, we first investigated the effect of various phospholipid bilayer systems and model raft mixtures on the activity of Na^+,K^+-ATPase. The lipid bilayer systems were chosen so as to be able to explore the effects of various membrane physical properties on enzyme activity, such as hydrophobic matching, curvature elastic stress, fluidity, and heterogeneity. The main goal was to study the effects of hydrostatic pressure and to correlate the structure and conformational properties of the lipid matrix to the function of the enzyme upon pressurization. To this end, enzyme activity studies were performed over a wide pressure range, varying from ambient pressure up to about 2 kbar. Moreover, the conformation and phase state of the pure lipid and reconstituted proteolipid systems were studied by the Laurdan fluorescence technique. In fact, the lipid matrix turned out to have a drastic effect on the function of the protein. Interestingly, in most cases, a multiphasic effect of pressure on the activity of the enzyme was found.

EXPERIMENTAL PROCEDURES

Reagents. The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), and sphingomyelin (SM) (brain, porcine) were obtained from Avanti Polar Lipids. 6-Dodecanoyl-2-dimethylamino-napthalene (Laurdan) was purchased from Molecular Probes, Inc. (Eugene, OR), and bio-beads SM-2 adsorbent was from Bio-Rad Laboratories (München, Germany). Chloroform was obtained from Roth (Carl Roth GmbH, Karlsruhe), and pyruvate-kinase/L-lactate dehydrogenase (PK/L-LDH) (from rabbit muscle) was obtained from Roche (Mannheim, Germany). Octaethylene glycol monododecyl ether ($C_{12}E_8$) was obtained from Fluka (Buchs, Switzerland), and adenosine 5′-triphosphate (ATP), 5-cholesten-3β-ol (cholesterol), β-nicotinamide-adenine dinucleotide (NADH), ethylenediaminetetraacetic acid (EDTA), magnesium dichloride ($MgCl_2$), sodium chloride (NaCl), potassium chloride (KCl), imidazole, Bis-Tris, hydrochloric acid (HCl), trichloroacetic acid (TCA), phosphoenolpyruvic acid, monopotassium salt (PEP), and bovine serum albumin were all obtained from Sigma-Aldrich (München, Germany).

Isolation and Purification of Na^+,K^+-ATPase. Na^+,K^+-ATPase was isolated from two main sources, rabbit and pig kidneys. In the context of this work, in the first step, an enrichment of the enzyme was accomplished in plasma membranes isolated from rabbit kidney outer medulla under the guidance of Professor Dr. E. Kinne-Saffran (Max-Planck Institute of Molecular Physiology, Dortmund). With this material in hand, initial pressure-dependent activity measurements have been carried out (*42*). In a second step, with the assistance of Professor Dr. J.A. Mignaco and Professor Dr. C.F.L. Fontes (Federal University of Rio de Janeiro, Rio de Janeiro), the enzyme was isolated from the pig kidney with SDS activation of the enzyme. The aim of this large-scale preparation was to obtain enough material for a series of biophysical experiments on reconstituted vesicle preparations using different lipid bilayer systems.

Enrichment of Na^+,K^+ -ATPase from Rabbit Kidneys in the Plasma Membrane. Na^+,K^+-ATPase was enriched in the membrane-bound form from rabbit kidney outer medullary tissue. To obtain renal tissue, white New Zealand rabbits (3 kg, either sex) were used. After cervical dislocation and bleeding, the kidneys were removed and placed in ice-cold ST-buffer (250 mM sucrose and 10 mM triethanolamine at pH 7.4, adjusted with HCl). The medulla tissue was carefully excised from the cortical tissue using a Stadie−Riggs tissue slicer (Thomas Scientific, U.S.A.). Then, 6 g of tissue was homogenized in 35 mL of ST-buffer in a Waring blender using an adaptor suitable for small volumes. Homogenization was performed in the cold (4 °C) twice for 30 s at full speed with an interval of 60 s. The resulting homogenate was poured through cheesecloth to remove any unbroken tissue. A series of centrifugation steps followed in which cell membranes were separated from nuclei, mitochondria, and the endoplasmic reticulum. The final pellet consisted of a firmly packed dark brown pellet overlaid by a loosely packed, whitish material, which could be separated by swirling it off. This fraction is termed the plasma membrane fraction. By this method, Na^+,K^+-ATPase was enriched about 6-fold (*38*).

Isolation of Na^+,K^+-ATPase from Pig Kidneys. The isolation was carried out in two steps. (1) Fresh pig kidneys (50−100 pieces from a local slaughterhouse) were obtained and placed on ice. The kidneys were cut lengthwise, and the inner medulla was removed and discarded. The pink and red tissue of the outer medulla was carefully dissected and placed in a solution containing 30 mM histidine and 250 mM sucrose at pH 7.3 (HS solution) at 20 °C (1 vol. buffer per vol. tissue) and subsequently placed on ice. The tissue was homogenized by pressing the material through a Yeda-Press, applying pressure with our hands. The resulting thick, red tissue suspension was mixed with 25 mM imidazole, 250 mM sucrose, and 1 mM EDTA at pH 7.4 (20 °C) (ISE solution), and mechanically homogenized at 4 °C with a Teflon pestle, 3 × 1500 RPM, in a tight glass homogenizer. After sequential low-speed centrifugations to clear up the supernatant from cell debris and mitochondria, microsomes were collected at 20,000 rpm × 40 min on an SS-34 rotor. The resulting pellet was suspended in a small volume of ISE, and SDS was added for activation of the enzyme, as described by Norby et al. (*43*), modified from Jorgensen (*44*). ATPase activity (*45*) and/or K-dependent pNPPase (*p*-nitrophenol-phosphomonoesterase assay) activity was measured. After the SDS-activation step, the mixture was centrifuged 40 min × 100,000*g* to and re-suspended on ISE buffer to remove excess detergent. This wash was repeated five times, and the final pellet was diluted on a very small amount of ISE solution (1 mL/1.5 g of the original tissue). One sample was used to determine protein purity and activity. The protein was stored at −20 °C until use. Using this protocol, an enzyme with very high purity was obtained (99.8% of ATPase activity was inhibited by 1 mM ouabain).

Purity was further confirmed by SDS−PAGE and Western-blot analysis. A DSC thermodiagram of SDS-activated Na^+,K^+-ATPase was also obtained (data not shown). The DSC scan shows, in good agreement with other results from other preparations (*46*, *47*), a DSC peak with a maximum around 58 °C. At this temperature, partial unfolding of the α- and β-subunits of Na^+,K^+-ATPase takes place (*47*).

Solubilization and Reconstitution of Na^+,K^+-ATPase. Detergent solubilization is required as a first step for the reconstitution of membrane proteins (*48*, *49*). A detergent that has been used with great success in Na^+,K^+-ATPase solubilization is octaethylene glycol monododecyl ether ($C_{12}E_8$), and different works showed that Na^+,K^+-ATPase previously treated with sodium dodecyl sulfate (SDS) can be solubilized with $C_{12}E_8$ in an active form, where most of the kinetic and conformational properties of the enzyme are preserved after solubilization (*50*, *51*).

Few methods are known for obtaining the removal of detergents, based on the detergent that is used, the rate of detergent removal, the chosen lipids, the molecular behavior of the protein, and the ionic conditions. A quite simple method that has proved successful involves solubilization of the protein in a suitable detergent (in this case $C_{12}E_8$) and its reconstitution with appropriate soluble lipids, in the presence of a small amount of adsorbent Bio-Beads (SM2) that specifically adsorb the detergent (*52*, *53*).

Solubilization was carried out at 4 °C by the rapid mixing of Na^+,K^+-ATPase membrane fragments with $C_{12}E_8$. A ratio of 1 mg enzyme /1 mg detergent was used. The non-solubilized parts were removed by centrifugation at 4 °C for 1 h at 100,000*g*. The supernatant was taken for further experiments. For reconstitution, the solubilized protein at 1 mg/mL was mixed with a suspension of phospholipids (DMPC, DOPC, the model raft mixture POPC/SM/Chol 1:1:1 and DOPC/DOPE 3:2) that was solubilized in the same detergent as the protein.

Detergent-solubilized phospholipids were prepared just prior to the reconstitution experiment. The phospholipids were dissolved in chloroform, with the proper amount of the fluorophore Laurdan, and dried first under a stream of N_2 gas and then under vacuum for 24 h, until all traces of chloroform evaporated. The dried lipids were resuspended in the detergent solution by vortexing, sonification for 5 min, and 4−5 freeze−thaw operations (frozen in liquid nitrogen and heated at 70 °C in a water bath) (*53*). The reconstituted systems contained ∼1−2 mol % of the protein.

Before reconstitution of the protein, it was essential to determine the proper amount of detergent to solubilize the phospholipid. An indication of the proper amount of detergent is given for the $C_{12}E_8$ concentration where the main phospholipid phase transition is abolished. The *GP* data exhibited that for the detergent to lipid ratio of 0.39, no such phase could still be detected, indicating that the lipid bilayer system had been dissolved with *GP* values around zero, typical for fluid-mixed micellar systems. The procedure for the reconstitution of the membrane transporter involves the removal of detergent from the solution containing the solubilized protein and the chosen lipids. Both lipids and the protein are present in the detergent solution as mixed lipid−detergent and protein−lipid−detergent micelles, respectively. The detergent was removed over a period of ∼4−5 h by the addition of small amounts (1 g per 5 mL solubilized protein−lipid solution) of adsorbent Bio-Beads (SM2) (*53*).

Measurement of Enzyme Activity. Na^+,K^+-ATPase activity was determined at 37 °C in an assay medium containing 20 mM HEPES-Tris buffer (pH 7.4), 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, and 3 mM ATP, in the absence or presence of 2 mM ouabain. After 30 min of incubation, the samples were put into a boiling water bath for 2 min to stop the reaction. After centrifugation of the samples, the phosphate in the supernatants was determined according to Fiske and Subbarow (*45*). The protein concentration was determined by the method of Lowry et al. after the precipitation of the samples in ice-cold 10% trichloroacetic acid, using unfractionated bovine serum albumin as the standard (*54*).

ATPase activity was also measured with the PEP/PK/LDH coupled assay method and NADH consumption followed by its fluorescence decrease at 460 nm. Within the time and pressure range covered, the assay is also pressure stable (*34*, *36*). The medium (total volume of 3 mL) contained 130 mM NaCl, 20 mM KCl, 0.1 mM EDTA, 4 mM $MgCl_2$, 3 mM phosphoenolpyruvate (PEP), 0.4 mM NADH, 50 mM Tris at pH 7.4, 48 μg of PK-L-LDH, and 200 μL of Na^+, K^+-ATPase (1 mg/mL). The reaction was started by adding 3 mM ATP, and the consumption of NADH was followed by its fluorescence decrease at 460 nm (see below).

Fluorescence Spectroscopic Measurements. All fluorescence spectroscopic measurements were carried out on a K2 multifrequency phase and modulation fluorometer (ISS, Inc., Champaign, IL). The fluorometer uses a xenon arc lamp as the light source. The decrease in NADH fluorescence intensity observed at 460 nm with time was measured in a high-pressure cell with quartz windows with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Temperature was controlled to ± 0.1 °C by a circulating water bath. All measurements were carried out at $T = 37$ °C. Pressure was controlled with an APP automated pressure control system (APCS, Ithaca, NY) and was applied with a ramp of ∼500 bar min^{-1}. For determining the generalized polarization function of the fluorophore, control measurements showed that no additional fluorescence changes took place after 15 min at each pressure and after 10 min at each temperature step.

The phase state of the lipid membrane systems was determined by the Laurdan fluorescence technique. The emission spectrum of the environmentally sensitive fluorescence probe 6-dodecanoyl-2-dimethyl-aminonapthalene (Laurdan) was used to scrutinize the phase behavior of the lipid bilayer system (Figure 1). Laurdan is a naphthalene-based amphiphilic molecule (*55*). Its fluorescence excitation and emission spectra are very sensitive to the polarity and dipolar dynamics of the environment (*56*, *57*). The quantum yield of Laurdan is much higher in the membrane than in aqueous solution. When inserted into a membrane, Laurdan aligns its lauroyl tail with the lipid moiety and locates its naphthalene ring near the phospholipid glycerol backbone (Figure 2). Studies using phospholipid vesicles demonstrated that Laurdan is sensitive to the dynamics as well as the polarity of the surrounding membrane. Therefore, Laurdan can distinguish whether a membrane is in a gel or a liquid-crystalline phase state (*58*). When Laurdan is embedded in a lipid membrane, it exhibits a 50 nm red shift of the emission spectrum as the membrane changes from the gel

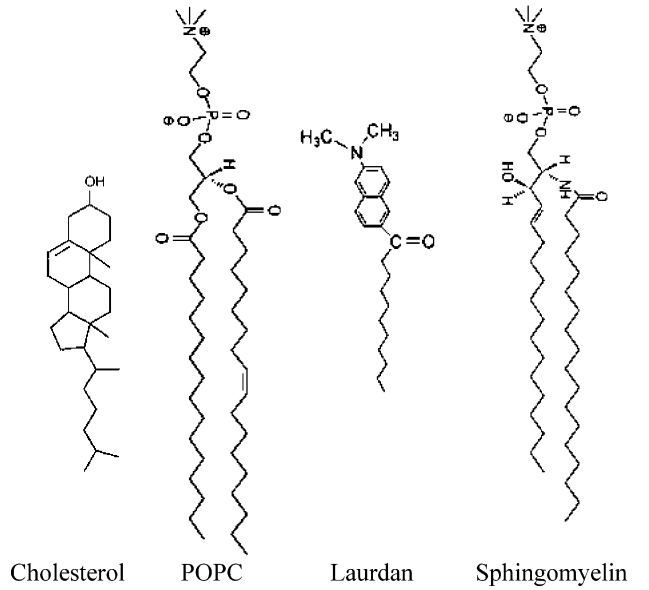

FIGURE 1: Chemical structures of the ternary system of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and sphingomyelin (POPC/SM/Chol) and the location of the fluorescence probe 6-dodecanoyl-2-dimethyl-aminonapthalene (Laurdan) in the lipid bilayer system.

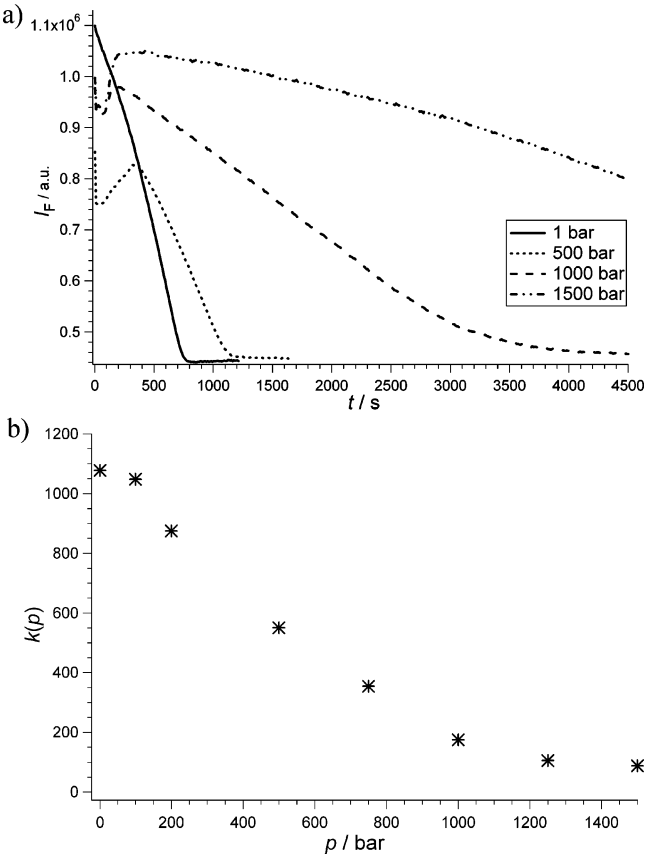

FIGURE 2: Activity of Na^+, K^+-ATPase from rabbit kidney enriched in the plasma membrane. (a) Decrease in NADH fluorescence intensity (I_F, in arbitrary units) at 460 nm and selected pressures (T = 37 °C). (b) Rate of NADH fluorescence intensity decay (proportional to enzyme activity), $k = |\Delta I_F/\Delta t|$, as a function of pressure at 37 °C.

into the liquid-crystalline phase. The spectroscopic properties of Laurdan in membrane studies are often described by its general polarization value, *GP*, defined as $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$ (*58*). I_{440} and I_{490} are the fluorescence intensities measured at the emission maxima (excitation *GP*) of Laurdan, characteristic of the gel and the liquid-crystalline phases, respectively. Hence, *GP* values range from −1 to +1. Within phase-coexistence regions, *GP* values exhibit values typical for fluid (liquid-disordered) and gel-type (liquid-ordered) domains.

Lipid stock solutions were prepared in chloroform. Laurdan was dissolved in chloroform at a concentration of 1 mmol/L. Vesicles containing pure phospholipids or the desired molar ratios of POPC, SM, and Chol were prepared together with the Laurdan fluorophore. After co-dissolving the lipids and fluorophore, the solvent chloroform was removed by a flow of nitrogen gas. Then the samples were dried under high vacuum pumping for several hours to completely remove the remaining solvent. The remaining dry film was then resuspended in water, vortexed, and sonicated for 15 min in a bath-type sonicator (Bandelin SONOREX RK100SH). Finally, several freeze−thaw cycles were applied to achieve a better homogeneity of the vesicle preparation. The final concentration of lipid vesicles in the samples used for the fluorescence measurements was 0.3 mmol/L and that of the fluorescent probe was ∼0.5 μmol/L. The final vesicle solution contained a 1:550 fluorophore to lipid mixture on a molecular basis.

RESULTS

Activity of the Naturally Derived Na^+,K^+-ATPase System. We first measured the activity of Na^+,K^+-ATPase from rabbit kidneys, enriched in its partially natural plasma membrane environment, by determining the decrease of NADH fluorescence at 460 nm with time as a function of hydrostatic pressure. To this end, we determined the intensity decay at the emission maximum of NADH at 460 nm. As an example, Figure 2a presents the fluorescence intensity data of NADH at 460 nm at selected pressures at 37 °C. At ambient pressure, the enzymatic reaction is completed after about 15 min. Our data clearly show that increasing pressure leads to a decrease in the reaction rate. Moreover, the measurements show that the activity of Na^+,K^+-ATPase is reversibly inhibited by pressures below 2 kbar. Enzymatic activity is completely recovered after a 15 min incubation at 1 bar after pressurization up to 2 kbar for more than 1 h. On the contrary, the activity of Na^+, K^+-ATPase was not recovered after incubation at higher pressures.

The activity plots $I_F(t)$ exhibit a rather complex behavior with a first fast increase and then a more or less slow decay of activity upon pressurization. This might indicate that the pressure-induced reaction involves two or more rate-limiting steps, which certainly is not unexpected because the ATPase reaction proceeds via several intermediate steps, which might be sensitive to pressure if significant changes in volume are involved. However, the first slight initial increase of activity might also be due to changes in lipid morphology. It may be assumed that the naturally derived lipid membrane preparation consists of flat and bent vesicular topologies. Upon pressurization, part of the lipid topologies might rearrange, which could lead to an increase in the exposure of active sites to the solution, thus enhancing enzymatic activity.

Figure 2b presents data of Na^+,K^+-ATPase activity, defined as $k(p) = |\Delta I_F/\Delta t|$, as a function of pressure at

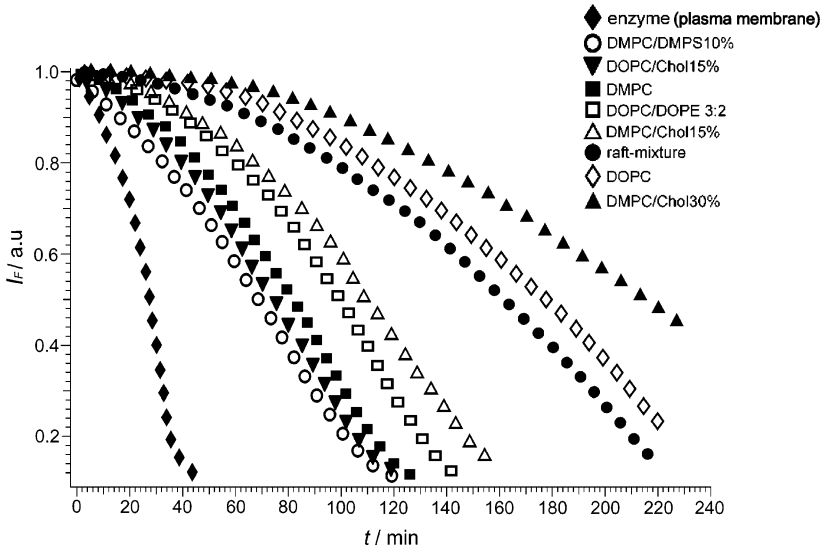

FIGURE 3: Comparison of the activity of plasma membrane-enriched Na^+,K^+-ATPase and Na^+,K^+-ATPase reconstituted into different lipid systems as determined by the NADH fluorescence intensity decay at 460 nm (at 1 bar and $T = 37$ °C).

37 °C, obtained from a linear regression method (neglecting the initial phase) of the individual measurements, such as those shown in Figure 2a. Clearly, $k(p)$ decreases approximately exponentially with increasing pressure. Hence, the plot of ln(k) versus pressure is essentially linear at longer timescales, thus allowing for the calculation of an apparent activation volume of the pressure-induced inhibition reaction, which amounts to $\Delta V^{\neq} = 47.1 \pm 2.3$ mL·mol^{-1}, a surprisingly high activation volume. In comparison, the activation volume of an organic model reaction for the hydrolysis of ATP, the hydrolysis of the acetyl phosphate dianion, is negative and gives -19 mL·mol^{-1} (*59*).

Generally, the following mechanisms of the inhibition of Na^+,K^+-ATPase activity by hydrostatic pressure could play an important role: (i) an increased packing and order of the lipid membrane hinders the required conformational changes of the enzyme, (ii) large volume changes of the enzyme during the enzymatic reaction, which may be independent of the lipid phase state, (iii) subunit dissociation, and (iv) a decrease of substrate binding because of a positive activation volume. As has been shown by many studies on phospholipid bilayer model systems, upon a pressure increase of a few hundred bars, the acyl-chain conformational order drastically increases, and various fluid−gel coexistence regions and gel phases may be induced (*21*, *22*), which might hinder conformational transitions associated with rate-limiting steps of the Na^+,K^+-ATPase reaction. As the plasma membrane consists of a very complex mixture of dozens of different lipids, no clear-cut pressure-induced fluid-to-gel transition pressure region is expected. We note that in lipid mixtures of binary and ternary phospholipid mixtures including model rafts mixtures, transition pressures to an overall ordered state of about 2 kbar are observed at ambient temperatures (*60*). This would be in accord with the findings of De Smedt et al. (*36*), who suggested that inhibition by pressure is due to a shift in the melting temperature of the lipid aliphatic chains. They measured the activity of Na^+,K^+-ATPase at different temperatures up to 600 bar and found that the break point in activity is in good agreement with the pressure shift for the melting transition in phospholipids and aliphatic chains. Because of the relatively low pressures applied, only a reversible inhibition of the enzyme was observed.

Activity of Pig Kidney Na^+,K^+-ATPase in Reconstituted Artificial Lipid Bilayer Systems. In the next step, we reconstituted the enzyme in different kinds of lipid bilayer systems, differing in chain length, degree of unsaturation, and phase state: DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; gel/fluid transition temperature at $T_m = 23.5$ °C); DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; $T_m = -20$ °C); DOPC/DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine) in 3:2 molar ratio; and the model raft mixture POPC/SM/Chol (1:1:1 molar ratio) (POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; Chol, cholesterol; SM, sphingomyelin). According to the phase diagram of the ternary system POPC/SM/Chol as given in Almeida et al. (*61*), the 1:1:1 mixture has coexisting l_o and l_d domains at ambient temperature.

Enzyme Activity in Various Reconstituted Lipid Systems at 37 °C. The enzyme activity of all reconstituted systems was first measured by NADH fluorescence decrease at $T = 37$ °C and ambient pressure. The data shown are normalized to the maximum intensity values given at $t = 0$. As can be clearly seen in Figure 3, the activities for all of the reconstituted systems are lower than the activity of the enzyme enriched in the plasma membrane. In the case of the reconstituted systems, depending on the lipid bilayer systems (*29*, *53*, *62*−*66*) and because of different protein concentrations upon reconstitution, a retardation of the reaction by a factor of 4−7 is observed. The reduction in intensity is also due to the enzyme not being oriented inside-out but rather non-oriented in the lipid bilayer model membrane systems. Most of the activity−time curves suggest a two-phasic or a continuous decay with an initial slower component, which might, at least partially, be due to the diffusion-limited reaction conditions after the addition of the enzymatic essay.

When a bilayer is made of monolayers with nonzero spontaneous curvature, it becomes subject to a built-in frustration termed curvature stress field. The stress can be released locally by changes in the local molecular composition, for example, by the binding/insertion of a protein. To

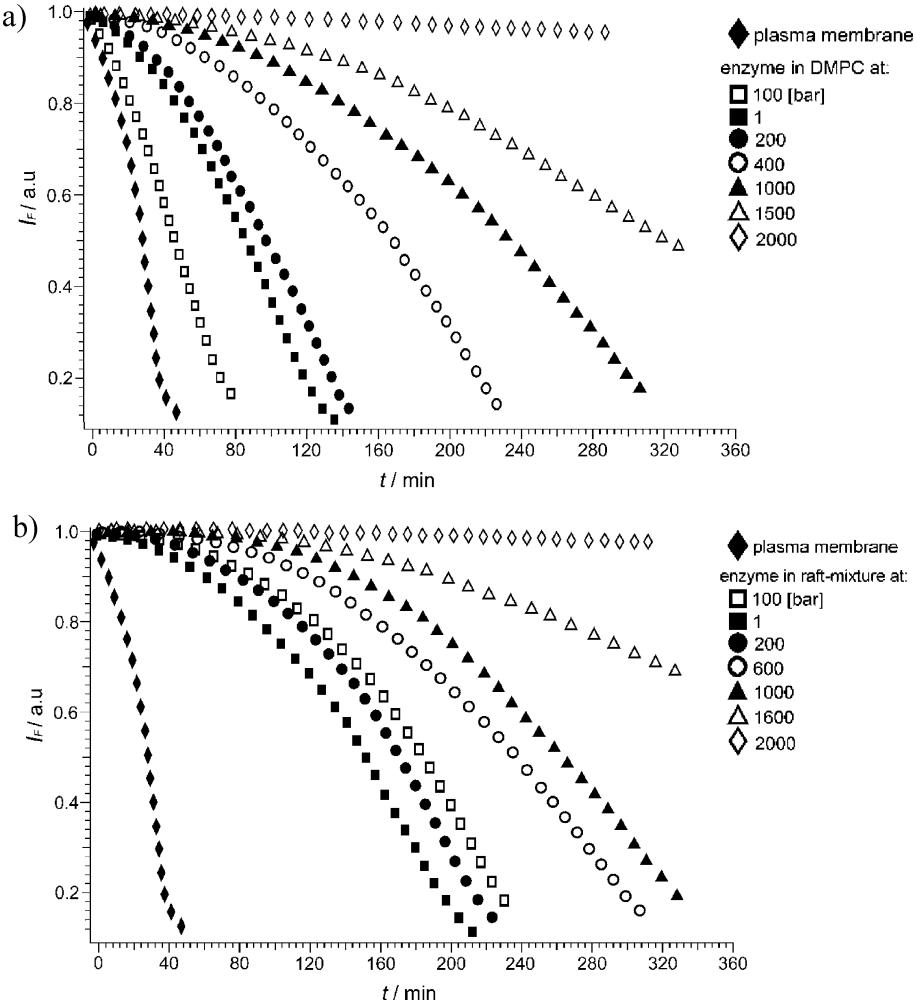

FIGURE 4: Decrease of NADH fluorescence intensity (460 nm emission wavelength) at 37 °C for (a) the enzyme reconstituted into DMPC and (b) the POPC/SM/Chol 1:1:1 model raft mixture for selected pressures in comparison to the plasma membrane-enriched Na^+,K^+-ATPase data.

also study the effect of an increase of the curvature elastic stress of the lipid membrane on Na^+,K^+-ATPase activity, reconstitution into a DOPC/DOPE 3:2 mixture was carried out. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are both zwitterionic lipids with similar chemical structures. However, PE lacks the three methyl groups on its head group moiety, and as a consequence, it has a much greater propensity of forming inverted (type II) phases. Adding DOPE to DOPC vesicles increases the tendency for curvature toward water and hence increases the local lateral stress. For the type II lipid amphiphile DOPE, the total stored curvature elastic stress for each DOPE molecule in a flat monolayer has been determined to be about 1 k_BT (*67*, *68*). The activity curve at 37 °C and ambient pressure are displayed in Figure 3 as well.

Pressure Dependence of Enzyme Activity at 37 °C in Various Lipid Bilayer Systems. The activity of the enzyme reconstituted into DMPC, DOPC, the model raft, and DOPC/DOPE mixture has also been measured in the pressure range from ambient pressure up to ~2 kbar at 37 °C. All intensity data were then normalized to their initial values. Two examples of the measurements carried out are shown in Figure 4. For both systems, DMPC and the model raft mixture, we can clearly see the inhibition of activity upon pressurization and, in the case of DMPC, a multiphase time dependence. Figure 5a−d shows the activities in terms of $|\Delta I_F/\Delta t|$ as a function of pressure for all reconstituted lipid systems studied. Similar to the enzyme enriched in the plasma membrane, for all of the reconstituted systems, a drastic decrease in activity is observed at high pressure. At pressures of about 2.0−2.2 kbar, enzyme activity decreases to zero within the time range of the experiment (~6 h). However, the rate of the pressure-induced decrease of the enzyme activity depends on the specific properties of the lipid bilayer system.

Very intriguing is the observation that in the low-pressure region of DMPC and DOPC an increase in enzyme activity is observed (of about 60−100%) with a maximum activity at about 100 bar for DMPC and DOPC bilayers (Figure 5a and b). Above 100−200 bar, this maximum is followed by a steep decrease in activity up to about 600−800 bar, where a more or less broad plateau value is reached, until the enzyme activity decreases to zero around 2 kbar for all reconstituted systems measured.

The 60−100% activation observed at low pressures may be related to a decrease in the volume of the ATPase complex induced by compression, which drives the system toward a negative activation volume of the ATPase reaction. This effect could also be related to a change in the lipid−protein system, which at these low pressures seems, however, to be

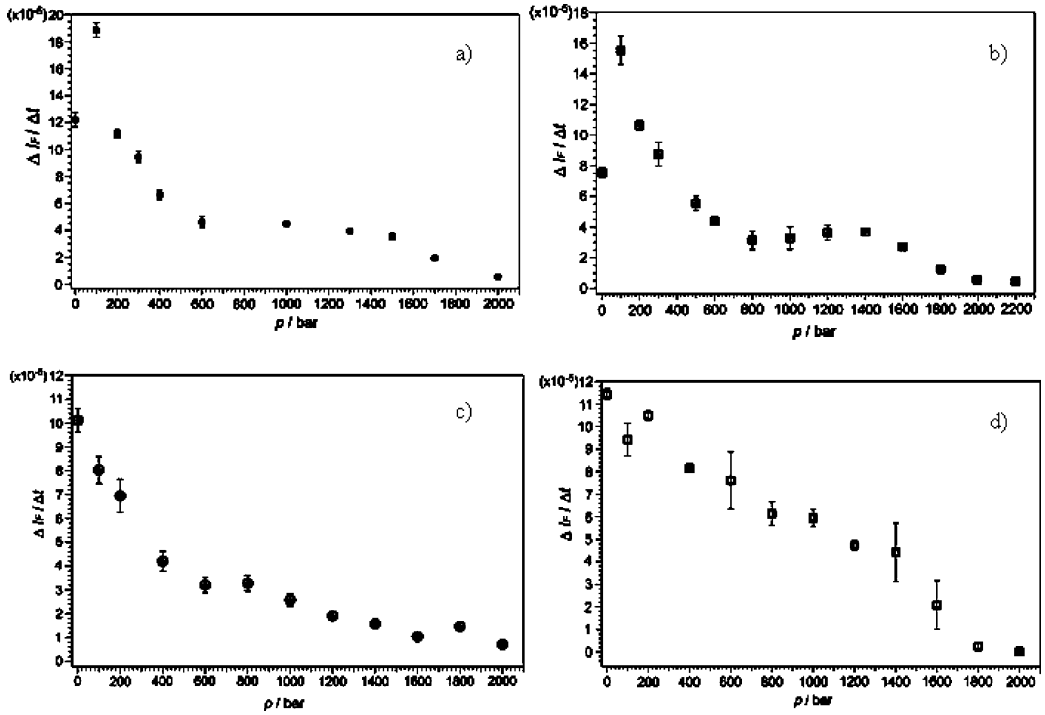

FIGURE 5: Modulus of the rate of NADH fluorescence intensity decrease $\Delta I_F/\Delta t$ (as obtained from the long-time tail of the fluorescence intensity data, $I_F(t)$), for selected reconstituted lipid bilayer systems: DMPC (a), DOPC (b), DOPC/DOPE 3:2 (c), and the model raft mixture POPC/SM/Chol 1:1:1 (d) ($T = 37$ °C).

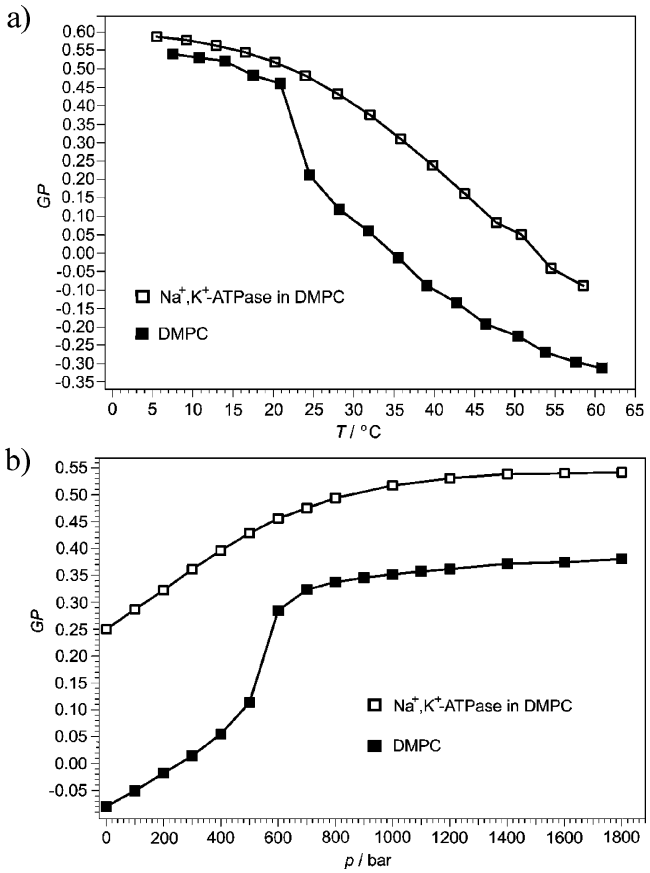

FIGURE 6: (a) Temperature dependence of Laurdan *GP* values of DMPC vesicles without and with incorporated Na^+,K^+-ATPase. (b) Pressure dependence (at $T = 37$ °C) of Laurdan *GP* values of DMPC vesicles without and with incorporated Na^+,K^+-ATPase.

less likely. An increase of 100 bar would only lead to a slight increase in lipid acyl chain length (ca. 0.1 Å) and packing, and no pressure-induced fluid-to-gel phase transition occurs at these rather low pressures (*22*, *69*). The subsequent decrease in ATPase activity as the pressure is raised above 100−200 bar may be induced by an increasing hydrophobic mismatch and eventually subunit rearrangement at higher pressures.

For the DOPC/DOPE 3:2 mixture (Figure 5c), there is, however, no acceleration at low pressures. We observe a rapid decrease of activity up to only 600 bar, followed by a less steep decrease of $|\Delta I_F/\Delta t|$. An even different scenario is observed for the effect of pressure on the enzyme activity in the model raft mixture (Figure 5d). In this case, a more or less linear decrease in activity is observed up to about 2 kbar, where the activity ceases.

From the exponentially decaying activity data in the pressure range from about 100 to 800 bar, an apparent activation volume $\Delta V^{\#}$ of the reaction may be obtained from the $\ln(|\Delta I_F/\Delta t|)$ versus p plots for the DMPC, DOPC, DOPC/DOPE mixture and for the raft mixture over the whole pressure range. Values for $\Delta V^{\#}$ of 78 ± 8 mL/mol, 70 ± 8 mL/mol, 49 ± 5 mL/mol, and 22 ± 4 mL/mol are obtained for the enzyme incorporated into DMPC, DOPC and DOPC/DOPE 3:2 bilayers, and the model raft mixture, respectively. The first two values are of similar magnitude compared to that of the enzyme in its natural plasma membrane environment (see above). For the higher component lipid mixtures, in particular the model raft mixture, smaller values for the activation volume are observed, which might be connected to the possibility that lipid sorting occurs upon pressure perturbation (see discussion below).

Pressure Dependence of GP Values of the Various Lipid Bilayers and Reconstituted Lipid−Enzyme Systems at 37 °C. To reveal whether the decrease in enzyme activity upon pressurization is related to pressure-induced lipid phase transitions, the temperature- and pressure-dependent Laurdan

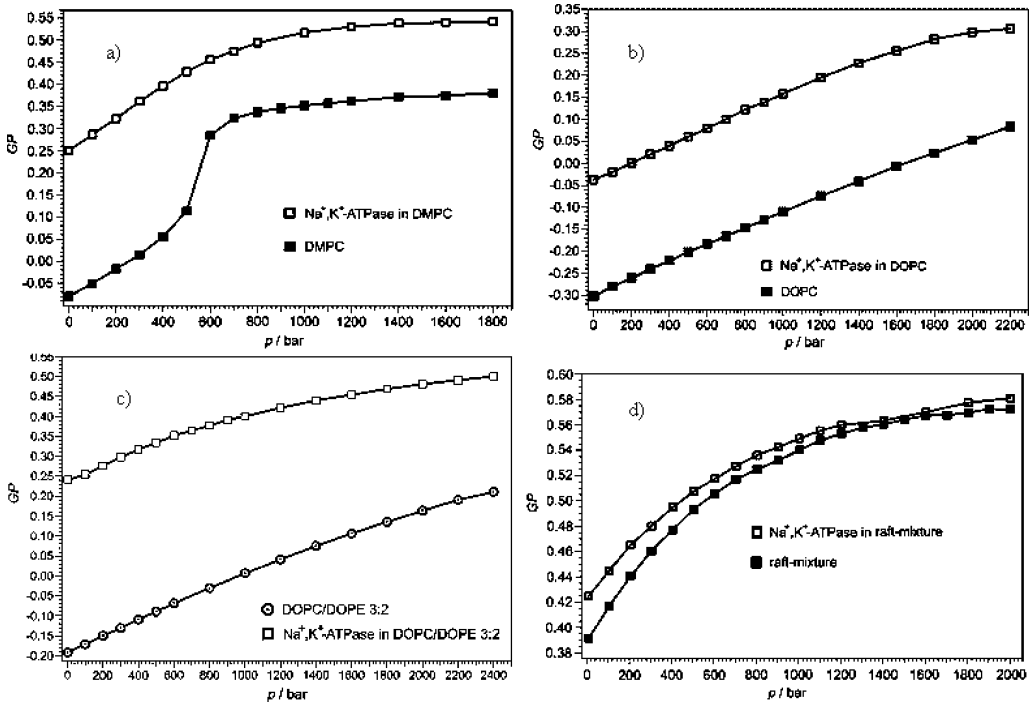

FIGURE 7: Pressure dependence of Laurdan *GP* values of DMPC (a), DOPC (b), DOPC/DOPE 3:2 (c), and the model raft mixture POPC/SM/Chol 1:1:1 (d) without and with incorporated Na^+,K^+-ATPase at $T = 37$ °C.

general polarization (*GP*) values of the various lipid and reconstituted proteolipid systems were measured as a function of temperature and pressure under equilibrium conditions. As explained above (Experimental Procedures), the *GP* values report on the phase state of the lipid bilayer system. The temperature-dependent measurements were carried out in the temperature range 7−70 °C, and the pressure dependent measurements were performed in the range 1−2000 bar at 37 °C.

We first focus on the one-component lipid bilayer systems. For Na^+,K^+-ATPase reconstituted in DMPC (Figure 6a) we can see that the *GP*(*T*) values first gradually decrease from 5 °C on with increasing temperature. The gel-to-fluid phase transition, as observed for pure DMPC bilayers around 23 °C, is not visible any more. Interestingly, the *GP* values of the reconstituted system are larger over the whole temperature range covered, indicating an increased order of the DMPC bilayer containing the enzyme. At 37 °C, the *GP* value of the reconstituted system is about 0.3, which is about 0.35 *GP* units above the *GP* value of the pure DMPC bilayer at that temperature. Figure 6b depicts the pressure-dependence data at 37 °C. For pure DMPC bilayers, the pressure-induced fluid-to-gel phase transition around 550 bar is indicated by a significant increase in *GP* values. For the reconstituted system, *GP* values are always much higher and increase steadily with increasing pressure, finally reaching *GP* values typical for densely packed gel-like lipids (0.55−0.6) at pressures above about 1200 bar.

The corresponding data for pure DOPC and Na^+,K^+-ATPase reconstituted into DOPC bilayers are depicted in Figure 7. At 37 °C, *GP* values of −0.3 are measured, indicating a highly conformational disorder for this system consisting of cis-unsaturated lipid chains. Because the main transition temperature T_m of DOPC is approximately −20 °C, no temperature- or pressure-induced phase transition is observed for this lipid system in the temperature and pressure range covered. Similar to DMPC bilayers, the incorporation of Na^+,K^+-ATPase into DOPC leads to an increase in *GP* values of about 0.3 units. For both pure DOPC and the reconstituted DOPC system, the *GP* value increases steadily with increasing pressure. Around 2 kbar, *GP* values of about 0.30 are reached. *GP* values of this order are indicative of a medium high conformational order parameter of the acyl chains.

For the POPC/SM/Chol 1:1:1 model raft mixture (Figure 7), *GP* values are much higher. At 37 °C, the system consists of coexisting l_d and l_o domains, leading to an average *GP* value of ∼0.40. For this model raft mixture, reconstitution leads to only a minor increase in *GP* values, that is, the phase state of the membrane. Because the *GP* values hardly change, one could also conclude that the enzyme is embedded to a large extent in the l_d/l_o domain boundary regions. The pressure effect is shown in Figure 7. The *GP* values of the pure lipid mixture as well as of the Na^+,K^+-ATPase−POPC/SM/Chol system gradually increase with increasing pressure, reaching *GP* values of ∼0.55−0.65, typical of an all-ordered phase state at pressures of ∼1500 bar. The effect of the incorporation of Na^+,K^+-ATPase leads to a small but steady increase of the order parameter value over the whole pressure range. Such an effect was also observed with the incorporation of the peptide gramicidin (*31*).

DISCUSSION

In principle, one could think of several general mechanisms of Na^+,K^+-ATPase inhibition by hydrostatic pressure: (1) an increased packing and order of the lipid membrane that hinders (dynamic) conformational changes of the enzyme needed for function, (2) volume changes of the enzyme itself upon pressurization, independent of the lipid phase state, (3) subunit reorientation or dissociation of the enzyme, (4) unfolding and denaturation of the protein, and (5) a pressure-induced decrease of the substrate binding

reaction due to a positive activation volume. Certainly, as has been shown by many studies on phospholipid bilayer model systems, by increasing pressure by only a few hundred bars, the acyl-chain conformational order drastically increases, and a more or less broad gel−fluid coexistence region as well as several pressure-induced gel phases may be induced at higher pressures (*21*, *22*, *69*), which might hinder conformational transitions associated with the ATPase reaction. This would be in accord with the observations of De Smedt et al. (*36*), who suggested that inhibition by pressure is due to a shift in the melting temperature of the lipid aliphatic chains. Chong et al. (*34*) concluded that the decrease in the fluidity of the membrane caused by increased pressure might hinder the conformational changes that accompany the reaction steps and thus decrease the rate of the overall reaction. However, it was also found that high pressures up to 2 kbar might lead to a dissociation of the subunits of the Na^+,K^+-ATPase (*39*). The pressure dissociation of water-soluble oligomeric proteins in this pressure range is well documented in several instances (*22*, *41*).

We measured the pressure-dependent activity of Na^+,K^+-ATPase enriched in the plasma membrane, and then we reconstituted the enzyme in different lipid bilayer matrixes. In accord with previous studies, we observed that high hydrostatic pressure leads to an inhibition of the enzyme enriched in the plasma membrane (Figure 2). Below ∼2000 bar, the activity is still reversible. Above this pressure, the enzyme is irreversibly destroyed.

The activity of the enzyme reconstituted into DMPC, DOPC, the model raft, and the DOPC/DOPE mixture has been measured in the pressure range from ambient pressure up to about 2 kbar at 37 °C. Generally, similar to the enzyme activity in the natural plasma membrane, an increase in high hydrostatic pressure leads to a retardation of the activity of the enzyme reconstituted into the various lipid bilayer systems, and in most cases, a multi-phasic behavior is observed. Clearly, no two-state transition is observed as in the pressure-induced unfolding reaction of many water-soluble monomeric proteins (*22*, *23*, *32*, *33*).

Interestingly, in the low-pressure regime around 100 bar, a significant increase of the enzyme activity (by 60−100%) is observed for the enzyme reconstituted into DMPC and DOPC bilayers. Above 100−200 bar, this maximum is followed by a steep decrease of activity up to about 600−800 bar, where a more or less broad plateau value is reached, until the enzyme activity decreases to zero around 2 kbar for all reconstituted systems measured (Figure 5).

The increased activation at these rather low pressures of about 100 bar may, for example, be related to a marked decrease in the volume of the ATPase complex induced by compression, which drives the system toward a more negative activation volume of the ATPase reaction. The effect could also be related to a structural change in the lipid matrix, which, at these low pressures, seems to be less likely, however. An increase of 100 bar would lead to a slight (ca. 0.1 Å) increase only in average lipid acyl-chain length, and no pressure-induced fluid-to-gel phase transition occurs at these rather low pressures (*22*, *69*). A different scenario is observed for the effect of pressure on the enzyme activity in the model raft mixture. In this case, a more or less linear decrease in activity is observed up to about 2 kbar, where the activity ceases. Obviously, the coexistence of l_o and l_d domains and the possibility of lipid sorting in this lipid mixture lead to a reduced pressure sensitivity of the enzymatic reaction in the medium-pressure range. This is also in accord with the much smaller activation volume of $\Delta V^{\#} = 22$ mL/mol observed for this lipid mixture. By lipid sorting and specific adsorption of long-chain lipids at the protein's interface, the large hydrophobic mismatch at elevated pressures may be partially avoided. Lipid heterogeneity is thus able to enhance the pressure resistivity of membrane proteins. At pressures of about 1−2 kbar, model raft mixtures also reach an overall ordered conformational state at ambient temperatures (*31*, *60*) so that this protection effect ceases above about 1.4 kbar.

The subsequent decrease of ATPase activity as pressure is raised above 100−200 bar may be explained by an increasing hydrophobic mismatch and eventually subunit spatial rearrangement (e.g., by interdigitation of loop regions or stretching of α-helices) or even subunit dissociation when the hydrophobic mismatch reaches a critical level. Generally, upon pressurization, a decrease in the fluidity of the lipid bilayer with a concomitant decrease in protein conformational flexibility is expected. Also the thickness of the lipid bilayer increases, which would be expected to alter the environment of the protein segments at the protein/water and protein/lipid contact interface. For example, an increase in lipid thickness might partially cover the water-soluble region of the enzyme, which could lead to a retardation of the reaction. Such an effect could lead to separation in the contact interface of the membrane and protein and hence to reversible conformational changes of the protein between 1 and 2 kbar. The physiological Na^+,K^+-ATPase reaction involves two conformational transitions, $E_2 \rightarrow E_1$ and $E_1P \rightarrow E_2P$, which might both be pressure sensitive. If a positive volume change would be involved or induced by these conformational changes, this could also be one factor explaining the retardation of activity upon pressurization. Because there is no pressure-induced fluid-to-gel phase transition in DOPC and DOPC/DOPE bilayers in the whole pressure range covered, the common low plateau value of enzymatic activity reached around 600−800 bar for DMPC, DOPC, and DOPC/DOPE 3:2 speaks for such conformational transitions of the protein rather than for a fluid-to-gel lipid transition as the major retarding mechanism. From the exponentially decaying activity data in the pressure range from about 100 to 800 bar, an apparent activation volume $\Delta V^{\#}$ of the reaction can be obtained from the $\ln(\Delta I_F/\Delta t)$ versus p plots. The large values determined for $\Delta V^{\#}$ of ∼50−80 mL/mol (which correspond to the volume of about 3−4 water molecules) relates to the marked pressure sensitivity of the non-raft lipid−protein mixtures. Higher pressures above about 2.2 kbar irreversibly also destroy the enzyme structure, in the reconstituted systems, most likely due to dissociation and partial unfolding of the protein subunits. A detailed FRET study of the protein construct with various fluorescently labeled regions might reveal more mechanistic information.

CONCLUSIONS

The pressure-dependent activity of Na^+,K^+-ATPase enriched in the plasma membrane and reconstituted into different lipid bilayer matrixes has been determined. Irreversible inhibition of the enzyme enriched in the plasma membrane occurs above ∼2 kbar, and the enzyme seems to

be irreversibly destroyed. Enzyme activity also decreases to zero around 2 kbar for the reconstituted systems. Interestingly, in the low-pressure regime around 100 bar, a marked increase of enzyme activity (up to ~100%) is observed for the enzyme reconstituted into DMPC and DOPC bilayers. Such a pressure-induced activation at low pressures might be of interest in light of the function of the enzyme in deep sea environments at depths of 1−2 km, where a similar pressure range prevails. Upon further pressure increase, maximum activity is followed by a steep decrease up to about 800 bar, where a broad plateau value is reached, until the enzyme activity decreases to zero around 2 kbar. As revealed by complementary generalized polarization (*GP*) fluorescence measurements, conformational transitions of the protein and increasing hydrophobic mismatch rather than a fluid-to-gel lipid phase transition as the major retarding mechanism seem to be operative. Heterogeneity of the lipid matrix such as in raft mixtures slightly decreases the pressure sensitivity of the membrane protein at intermediate pressures. Finally, above ~2.2 kbar, irreversible changes of the protein conformation occurs, probably due to the dissociation of the subunits.

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