Stabilization of Na^+,K^+-ATPase Purified from *Pichia pastoris* Membranes by Specific Interactions with Lipids†

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ABSTRACT: Na^+,K^+-ATPase (porcine α_1/His_{10}*β_1 or human α_1/porcine His_{10}*β_1) has been expressed in *Pichia pastoris* and purified by Co^{2+}-chelate affinity resin chromatography, yielding about 80% pure, functional, and stable protein in a single step. The protein was eluted in nonionic detergents together with a phosphatidylserine. Size exclusion chromatography showed that the protein eluted in *n*-dodecyl β-D-maltoside is an α_1/β_1 protomer, whereas that in octaethylene glycol dodecyl monoether contains a mixture of α_1/β_1 protomer and higher order oligomers. The Na^+,K^+-ATPase activity (8−16 (μmol/min)/mg of protein) is similar in both detergents. Thus, the minimal functional unit is the α_1/β_1 protomer, and activity is unaffected by the presence of oligomeric forms. Screening of phospholipids for stabilization of the Na^+,K^+-ATPase activity shows that (a) acid phospholipids are required and phosphatidylserine is somewhat better than phosphatidylinositol and (b) optimal stabilization is achieved with asymmetric phosphatidylserines having saturated (18:0 ≥ 16:0) and unsaturated (18:1 > 18:2) side chains at *sn*-1 an *sn*-2 positions, respectively. In the presence of phosphatidylserine, cholesterol stabilizes the protein at 37 °C, but not at 0 °C. Cholesterol also increases the "apparent affinity" of the phosphatidylserine and stabilizes optimally in the presence of phosphatidylserines with a saturated fatty acyl chain at the *sn*-1 position. Ergosterol is a poor stabilizer. We propose that phosphatidylserine and cholesterol interact specifically with each other near the α_1/β_1 subunit interface, thus stabilizing the protein. These interactions do not seem to affect Na^+,K^+-ATPase activity.

P-type ATPases utilize the free energy of hydrolysis of ATP to actively pump cations across biological membranes. The kinetic mechanism of the P-type ATPases, involving specific cation-catalyzed phosphorylation and dephosphorylation reactions and E_1/E_2 conformational changes, coupled to the cation movements, is largely understood (*1, 2*). The molecular mechanism of active cation transport is also being clarified by the crystal structures of the sarcoplasmic reticulum Ca^{2+}-ATPase in different conformations (*3*−*9*). Determination of the molecular structure of other P-type ATPases such as the prototype Na^+,K^+-ATPase, or the closely similar gastric H^+,K^+-ATPase, remains a challenge. Structures of these pumps could reveal the molecular basis of cation selectivity and the roles of the β subunit (*10*) and, in the case of the Na^+,K^+-ATPase, regulatory FXYD proteins (*11*−*13*). Furthermore, structures may elucidate binding sites and effects of specific inhibitors such as cardiac glycosides on Na^+,K^+-ATPase or the K-competitive SCH28080 class of inhibitors of H^+,K^+-ATPase. Native renal or duck salt gland Na^+,K^+-ATPase preparations have been used for 2D crystallization and cryoelectron microscopy (*14, 15*). A structure of the purified recombinant N domain of Na^+,K^+-ATPase has been described (*16, 17*). A 3D crystal structure of the detergent-soluble renal Na^+,K^+-ATPase could be available in the not too distant future (P. Nissen, personal communication). In principle, crystal structures of recombinant P-type ATPases, particularly of mutants, isoforms, species variants, and complexes with other proteins (e.g., FXYD), should provide valuable additional insights, compared to those obtained from the structures of the native P-type pumps. As one example, a structure of a recombinant Ca^{2+}-ATPase expressed in *Saccharomyces cerevisiae* has been reported (*18*).

We have recently described purification of Na^+,K^+-ATPase, expressed in the methylotrophic yeast *Pichia pastoris*, as the *n*-dodecyl β-D-maltoside (DDM)[1]-soluble porcine α_1/His_{10}*β_1 subunit complex (*19, 20*), and also purification of an α_1/His_{10}*β_1/$FXYD_1$ complex (*21*). One important feature of *P. pastoris* is that it can grow to high densities and produce large amounts of membrane protein.

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[1] Abbreviations: DDM, *n*-dodecyl β-D-maltoside; $C_{12}E_8$, octaethylene glycol monodecyl ether; HPLC, high-performance liquid chromatography; Endo-H, endoglycosidase H; Ni−NTA, nickel−nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein 5-isothiocyanate; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPI, 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine]; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine]; DSPS, 1,2-distearoyl-*sn*-glycero-3-[phospho-L-serine]; SLPS, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-[phospho-L-serine]; DLPS, 1,2-dilinoleoyl-*sn*-glycero-3-[phospho-L-serine].

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Using Ni−NTA chromatography, approximately 1 mg of 30−50% pure porcine α_1/β_1 could be obtained from a 3 L culture of *P. pastoris*. The purity could be raised to about 80% by an additional size-exclusion HPLC step, but this was associated with loss of protein and variable stability of the eluted protein. A second important observation was the necessity to add dioleoylphosphatidylserine (DOPS) together with the DDM, in the washing and elution buffers, to obtain functional Na^+,K^+-ATPase. This fits well with a previous report that addition of phophatidylserine (PS) or other acid phospholipids is required to maintain activity of renal Na^+,K^+-ATPase, upon solubilization in $C_{12}E_8$ and separation on a size-exclusion HPLC column (*22*−*24*). In the case of the recombinant protein, the DOPS was found to protect specifically against rapid inactivation of Na^+,K^+-ATPase activity in an elution buffer containing K^+ and also after deglycosylation of the β subunit. It was proposed that the DOPS interacts specifically with the protein and that the same stabilizing interaction, near the α and β subunit interface, protects the protein in both K^+ and after deglycosylation. A third finding was that the protein purified in a medium containing DOPS and DDM is present as the α/β protomer, as judged by size-exclusion HPLC and sedimentation velocity.

The major objectives of the present work have been to define the specificity of the lipid−protein and detergent−protein interactions with the detergent-solubilized recombinant Na^+,K^+-ATPase and improve the protein purity, yield, and stability. Indeed, investigating the origin of destabilization of the protein in the detergent solution, and means to minimize destabilization, has proven to be both challenging and highly informative. The purified detergent-soluble Na^+,K^+-ATPase preparation is particularly well suited for detecting specific lipid effects on Na^+,K^+-ATPase activity and stability, measured as preservation of activity over time. By contrast, with few exceptions (*22*, *23*) previous studies on the effects of lipids on Na^+,K^+-ATPase have been done with native membranes or reconstituted lipid micelles and proteoliposomes. In these systems, because the protein is embedded in the lipid bilayer, it is difficult to distinguish between the effects of lipids acting as the physical environment of the protein and specific lipid−protein interactions or between the effects of lipid on Na^+,K^+-ATPase activity and protein stability (*25*, *26*). To investigate the effects of the detergent, the properties of the purified protein eluted in either DDM or $C_{12}E_8$ were examined. $C_{12}E_8$ was of interest because this detergent has been used extensively to solubilize native Na^+,K^+-ATPase for biochemical and biophysical characterization (*27*−*29*). Various oligomeric forms of native Na^+,K^+-ATPase dissolved in $C_{12}E_8$ have been described (α/β, $(\alpha/\beta)_2$, and $(\alpha/\beta)_4$) (*22*−*24*). Specific oligomeric forms of the protein might confer stability. Oligomeric forms might also be necessary for protein function, as discussed previously (*30*, *31*), although this is controversial.

We describe here improved procedures for purification and stabilization of porcine α_1/β_1 and human α_1/porcine β_1 complexes, evidence that the α_1/β_1 protomer is the minimal functional unit and, especially, evidence for specific stabilizing interactions of PS and cholesterol. In the Discussion, our findings are compared to the effects of phospholipids and cholesterol on Na^+,K^+-ATPase, which have been investigated extensively in the past, and the roles of annular versus nonannular lipids, discussed in the recent literature (*25*, *26*).

EXPERIMENTAL PROCEDURES

Media. YPD: 1% Bacto yeast extract, 2% Bacto-peptone, 2% dextrose; to solidify the medium, 2% Bacto-agar was added. YNB: 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 1% glycerol. BMG: 1.34% yeast nitrogen base without amino acids (Difco, Kansas City), 0.04% biotin, 0.1 M potassium phosphate buffer, pH 6.0, glycerol 1−3%. BMM: 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 0.5% methanol, 0.1 M potassium phosphate buffer, pH 6.0. Basal salt medium: (per liter) 26.7 mL of H_3PO_4, 0.93 g of $CaSO_4 \cdot 2H_2O$, 18.2 g of K_2SO_4, 14.9 g of $MgSO_4 \cdot 7H_2O$, 4.13 g of KOH, 40 g of glycerol. Trace element solution PMT1 (per liter): 6.0 g of $CuSO_4 \cdot 5H_2O$, 0.8 g of KI, 3.0 g of $MnSO_4 \cdot H_2O$, 0.2 g of $Na_2MoO \cdot 2H_2O$, 0.2 g of H_3BO_3, 0.5 g of $CaSO_4 \cdot 2H_2O$, 20 g of $ZnCl_2$, 65 g of $FeSO_4 \cdot 7H_2O$, 0.2 g of biotin, 1 mL of concentrated H_2SO_4, filter-sterilized and addition of 2 mL/L of basal salt medium. The trace salt solution is also added to the methanol feed supply at 2 mL/L.

Construction of the Clones pHIL-D2 (Porcine α_1/His$_{10}$ β_1 and Human α_1/Porcine His$_{10}$*β_1)*. A pHIL-D2 vector (Invitrogen) construct containing cDNAs encoding porcine α_1 (accession no. X03938) and porcine β_1 (accession no. X04635) with a 10-histidine tag at its N′ terminus (α_1/His$_{10}$*β_1) has been described previously (*20*). Human α_1 (accession no. X04297) in vector pNKS2 was a gift from K. Geering, Université de Lausanne, Switzerland. The coding regions were excised with *Xba*I and subcloned into the pHIL-D2 (human α_1/His$_{10}$*β_1), thus replacing the porcine α_1 insert.

Yeast Transformation, Selection, and Growth. SMD1165, a protease-deficient strain (his4, prb1) of *P. pastoris*, was transformed with 10 μg of *Not*I-linearized pHIL-D2 (porcine α_1/His$_{10}$*β_1 or human α_1/porcine His$_{10}$*β_1) construct. Preparation of spheroplasts and selection for His^+, Mut^s transformants and dot blot analysis, to screen for maximal copy number, were done as described (*19*, *20*).

Growth in Spinner Flasks and a Bioreactor. Initially, the cells were grown in an 8 L Bellco spinner flask, and methanol induction of Na^+,K^+-ATPase expression was done as described previously (*20*). Subsequently, transformants of *P. pastoris* were grown in a Bioflo-110 fermentor, or bioreactor (New Brunswick, NJ) with a volume capacity of up to 7 L. A 5 mL BMG starter was inoculated with the porcine α_1/His$_{10}$*β_1 or human α_1/porcine His$_{10}$*β_1 transformant and grown at 30 °C until the stationary state (36 h). This culture was then used to inoculate a 200 mL BMG starter. The latter was grown to OD_{600} 2−6 and was used as an inoculum for 3−8 L of BMG medium (with 3% glycerol) or basal salt medium plus trace elements (with 4% glycerol). Dissolved oxygen (dO_2) and pH (5.9) were controlled throughout the fermentation by manually changing the airflow rate and agitation and titrating the pH with 5% ammonium hydroxide. After ca. 36 h, the glycerol in the medium was exhausted, as confirmed by a relatively rapid increase of dO_2 to initial values. At this point the cell density reaches an OD_{600} of 60−70. Then a ca. 4 h feed batch stage was introduced in which 50% glycerol (15 (mL/L)/h) was added continuously and the dO_2 was maintained at 35−40%

initial dO_2. After the feeding was stopped, dO_2 rose to the initial level. The OD_{600} was 100−120. Protein expression was then induced at 25 °C, either by adding a bolus of 0.5% (v/v) methanol, three times daily, or an initial bolus of 0.5% (v/v) methanol and then a continuous methanol feed of 1−2 (mL/h)/3 L of culture. YNB to 1.34% plus biotin was also added (as in the BMG medium). After 2 days of induction, the cells were harvested by centrifugation, washed twice in sorbitol, 1.4 M, MOPS−Tris, 10 mM, pH 7.2, and EDTA−$4Na^+$, 1 mM, and frozen to −20 °C. The wet cell weight (WCW) at the end of 2 days was 70−100 g/L of medium.

Membrane Preparations. P. pastoris cells were broken with glass beads, and urea-treated membranes were prepared as described in ref *20*. Roughly 1 g of membrane protein was obtained per 100 g of cells. Pig kidney Na^+,K^+-ATPase was prepared as described in ref *32*.

Purification of Recombinant Na^+,K^+-ATPase. Membranes were homogenized with DDM/protein (2:1, w/w) in a medium containing NaCl, 250 mM, Tris−HCl, 20 mM, pH 7.4, imidazole, 5 mM, PMSF, 0.5 mM, and glycerol, 10%. The protein concentration was varied from 1 to 5 mg/mL and that of DDM adjusted accordingly from 2 to 10 mg/mL, respectively. The unsolubilized material was removed by ultracentrifugation. EDTA was added at 50−250 μM at protein concentrations of 1−5 mg/mL, respectively. The DDM-solubilized membranes were then incubated overnight at 4 °C with BD-Talon (Co^{2+}-chelate) beads, at a ratio of 100 μL of beads per supernatant from 10 mg of membranes. The beads were washed twice for 5 min at 0 °C by gentle mixing and aspiration of 5 equivalent volumes of a solution containing NaCl, 100 mM, Tris−HCl, 20 mM, pH 7.4, DDM, 0.2 mg/mL, or $C_{12}E_8$, 0.1 mg/mL, DOPS or SOPS, 0.05 mg/mL, or other lipids and cholesterol at 0.01 mg/mL, as indicated, glycerol, 10%, and imidazole, 10 mM. Protein was eluted by mixing the beads for 40 min at 0 °C with 1 equivalent volume of the same washing buffer containing imidazole, 150 mM. The eluted protein was stored at 0 °C. The protein was concentrated where indicated by ultrafiltration using Amicon-100 filters (Millipore). The filters were equilibrated with the elution buffer overnight before use.

When the protein was to be deglycosylated, after a 6 h incubation of the DDM-solubilized membranes with the BD-Talon beads, the beads were centrifuged at low speed and most of the supernatant was removed. Then Endo-H (100 units/10 mg of membrane protein) was added, and the beads were incubated further overnight at 0 °C. The beads were then washed, and the protein was eluted as above.

Phospholipids dissolved in chloroform were dried in a stream of nitrogen and dissolved at 5 mg/mL in $C_{12}E_8$, 10 mg/mL, or DDM, 20 mg/mL. Cholesterol was dissolved at 0.1 mg/mL in 1 mg/mL $C_{12}E_8$ or 2 mg/mL DDM.

Protein Determination. For convenience, the protein concentration of the α/β complex was usually determined by comparing Coomassie stain of the α subunit with that of a standard amount of pig kidney Na^+,K^+-ATPase. This method overestimates the true protein concentration by about 2-fold due to a difference in the purity of the test (recombinant ca. 80% pure) and standard (renal ca. 40% pure). The correction factor was found to be almost exactly 2-fold by a direct estimate of the purified recombinant protein concentration, using the Lowry assay, calibrated by amino acid analysis using BSA as a standard. Specific activities are calculated on the basis of the correct protein concentration.

SDS−PAGE and Size Exclusion HPLC. A 2−5 μg portion of recombinant enzyme or 40 μg of yeast membranes was separated on 7.5% or 10% polyacrylamide SDS−tricine gels (*33*). The gels were stained with Coomassie, scanned with an imaging densitometer (GS-690, BioRad), and analyzed using the Multi-Analyst software (BioRad). Size exclusion HPLC was done using a Superdex 200 column (300 × 10 mm) (Amersham Pharmacia Biotech) and an Ettan LC chromatography system. The protein was eluted at 0.5 mL/min, in a medium containing NaCl, 150 mM, Na^+−HEPES, 50 mM, pH 7.4, and $C_{12}E_8$, 0.1 mg/mL, or DDM, 0.2 mg/mL.

Na^+,K^+-ATPase Assays. Measurement of Thermal Stability. Na^+,K^+-ATPase assays on the purified enzyme were performed as described in refs *19* and *20*. Most Na^+,K^+-ATPase assays were done at 37 °C in a reaction medium containing 1 mM ATP + [γ-^{32}P]ATP. Assays at 0 °C were done with 2 μM ATP + [γ-^{32}P]ATP. Some later experiments were done with a colorimetric assay using malachite green dye to detect the phosphomolybdate (P_i Color Lock, Innova Biosciences). When the protein was prepared in DDM/PS, the reaction medium also contained DDM, 0.2 mg/mL, and DOPS, 0.05 mg/mL. When the protein was prepared in $C_{12}E_8$/PS, the reaction medium did not contain added $C_{12}E_8$/PS.

For measurement of thermal stability at 0 °C, aliquots were removed from the protein stored at 0 °C (over days) and Na^+,K^+-ATPase activity was measured at 37 °C. Otherwise, the protein was incubated at the indicated temperatures (usually 37 °C), and aliquots (5−10 μL) were removed after the indicated times (usually in minutes over 2 h) to ice-cold tubes containing 10 μL of the reaction medium. At the end of the incubation, aliquots were taken for measurement of Na^+,K^+-ATPase activity at 37 °C. The ^{32}P release was measured at three time points over 10−15 min, and the rate of Na^+,K^+-ATPase activity was calculated from the linear slope ± SE (range of SE 1−10%). Alternatively, in some thermal inactivation experiments the samples were assayed in duplicate, with values differing by less than 10%. For these experiments error bars do not appear (e.g., Figures 5, 7, and 8).

Ouabain binding to yeast membranes using [^{3}H]ouabain was done as in refs *19* and *20*.

Materials: BD-Talon metal affinity resin (catalog no. 635503) was obtained from Clontech. *n*-Dodecyl β-D-maltopyranoside, anagrade (catalog no. D310), and octaethylene glycol monododecyl ether, anagrade ($C_{12}E_8$) (25%, w/w) (catalog no. O330), were from Anatrace. Synthetic lipids: 1,2-Dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DMPS), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DPPS), 1,2-distearoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DSPS), 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DOPS) (catalog no. 830035), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (POPS), 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (SOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol (ammonium salt) (DOPI), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (catalog no. 850375) were obtained from Avanti Polar Lipids and stored

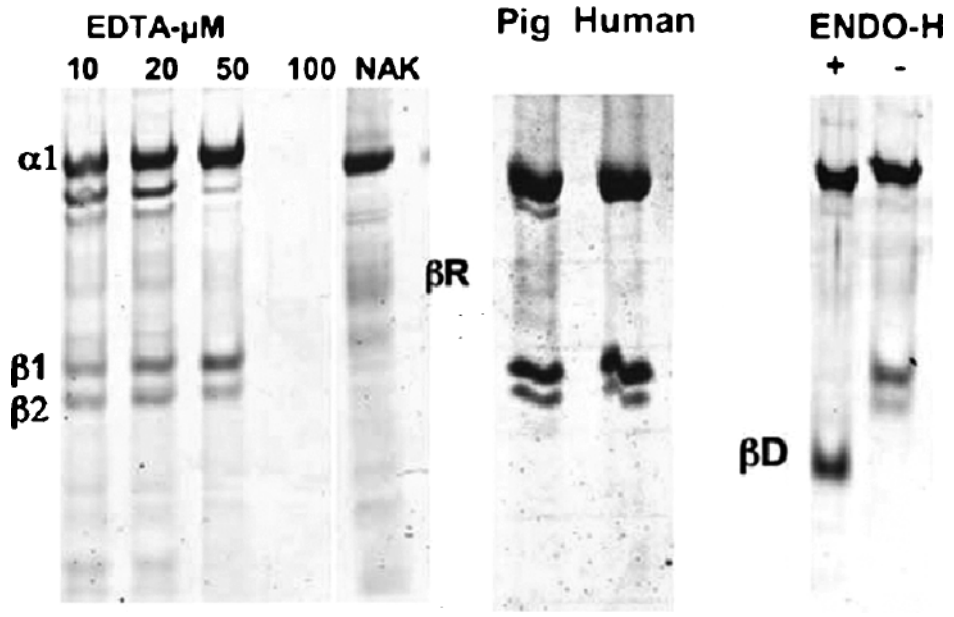

FIGURE 1: Purification of recombinant Na^+,K^+-ATPase (porcine $\alpha_1/His_{10}*\beta_1$ and human α_1/porcine $His_{10}*\beta_1$) by Co^{2+}–chelate chromatography. Each lane was loaded with about 5 μg of protein. Lanes 1–4: purification of porcine $\alpha_1/His_{10}*\beta_1$ bound to BD-Talon beads using indicated concentrations of EDTA (2 mg/mL DDM/1 mg/mL protein). Lane 5: NKA pig kidney enzyme. Lanes 6 and 7: comparison of purified porcine $\alpha_1/His_{10}*\beta_1$ and human α_1/porcine $His_{10}*\beta_1$. Lanes 8 and 9: porcine $\alpha_1/His_{10}*\beta_1$ treated or not treated with Endo-H, as described in the Experimental Procedures.

as chloroform solutions. Cholesterol and ergosterol were purchased from Sigma. [^{32}P]ATP and [^{3}H]ouabain were obtained from Amersham. Recombinant Endo-H (400 000 units/mg, catalog no. P0702S) was obtained from Bio Labs. All other materials were of analytical grade.

RESULTS

Expression and Purification of Na^+,K^+-ATPase (α_1/β_1). Porcine $\alpha_1/His_{10}*\beta_1$ or human α_1/porcine $His_{10}*\beta_1$ subunits were expressed as described in refs *19* and *20*. The cells were grown in either spinner flasks or a fermentor, and membranes were prepared and used for purification of the protein. The ouabain binding capacity was in the range 15–30 pmol/mg of protein.

The experiment in Figure 1 presents results of an improved purification procedure for the α_1/β_1 complex, which utilizes BD-Talon metal affinity resin (Co^{2+}-chelate), rather than Ni–NTA beads used previously (*20*). The purification of porcine $\alpha_1/His_{10}*\beta_1$ and human α_1/porcine $His_{10}*\beta_1$ complexes was essentially identical. The current procedure provides ca. 80% pure protein in a single batch step, without additional HPLC purification. The washing and elution buffers contained either $C_{12}E_8$ or DDM and a PS (DOPS or SOPS), as described below. The eluted protein consists of the α_1 subunit, two lightly glycosylated species of the β_1 subunit described previously in ref *20*, and contaminants. An important feature seen in Figure 1 was the inclusion of low concentrations of EDTA in the medium of the BD-Talon beads (lanes 1–3). This maneuver improves purity, reducing binding and elution of contaminant proteins, particularly those running just below the α subunit. With membranes at 1 mg/mL and DDM at 2 mg/mL the optimal concentration of EDTA was 50 μM. Note that it was necessary to carefully titrate the added EDTA, for excessively high concentrations (100 μM) remove Co^{2+} from the beads, and no protein was eluted (lane 4). Higher concentrations of DDM and membrane protein could also be used for the initial solubilization. In this case, higher concentrations of EDTA were used, in proportion to the concentration of membrane protein (e.g., 250 μM EDTA with 5 mg/mL protein/10 mg/mL DDM). As shown previously (*20*) and in Figure 1 the β subunit is easily deglycosylated by incubation overnight with Endo-H at 4 °C, thus producing a preparation consisting only of α and deglycosylated β subunit. In this particular experiment the contaminant band below the α subunit was not seen, and the protein was ca. 85% pure. The concentration of eluted protein was usually 0.1–0.3 mg/mL, and 15–25 μg was obtained from 10 mg of membrane protein.

Detergent Effects on Oligomeric Structure and Stability. Solubilization of *P. pastoris* membranes was always done using DDM, whereas washing of the BD-Talon beads and elution of protein could be done either with $C_{12}E_8$/PS or with DDM/PS.[2] The specific Na^+,K^+-ATPase activity at 37 °C, 8–15 (μmol/min)/mg of protein, was quite similar for the porcine $\alpha_1/His_{10}*\beta_1$ and human α_1/porcine $His_{10}*\beta_1$ complexes and were not significantly different for protein prepared with either $C_{12}E_8$/DOPS or DDM/DOPS (see Table 1). Nevertheless, striking differences in oligomeric state and absolute stability have been detected in $C_{12}E_8$ or DDM.

The oligomeric state of the purified α_1/β_1 complex was examined by size-exclusion chromatography (Figure 2). The human α_1/porcine $His_{10}*\beta_1$ complex was prepared in $C_{12}E_8$ (0.1 mg/mL)/SOPS (0.05 mg/mL) and concentrated to about 7 mg/mL. Aliquots were separated with the HPLC running buffer containing either 0.2 mg/mL $C_{12}E_8$ or 0.2 mg/mL DDM. The protein prepared with $C_{12}E_8$ and run in $C_{12}E_8$ showed a polydisperse pattern, with peaks corresponding to the α/β protomer (ca. 11 min), higher order oligomers (α/

[2] The standard concentrations of $C_{12}E_8$ and DDM used in the washing and elution buffers, 0.1 and 0.2 mg/mL, respectively, correspond to 186 and 391 μM and are close to 2-fold higher than the critical micelle concentration, 90 and 180 μM, respectively (*34*).

Table 1: Specific Na^+,K^+-ATPase Activity of the Porcine α_1/β_1 Complex Prepared in Either DDM or $C_{12}E_8$[a]

elution conditions	Na^+,K^+-ATPase activity, (μmol/min)/mg ± SE
DDM, 0.2 mg/mL, DOPS, 0.05 mg/mL	8.9 ± 0.6
$C_{12}E_8$, 0.08 mg/mL, DOPS 0.02 mg/mL	10.2 ± 1.2
$C_{12}E_8$, 0.2 mg/mL, DOPS, 0.05 mg/mL	9.6 ± 0.6
$C_{12}E_8$, 0.2 mg/mL, DOPS, 0.1 mg/mL	8.0 ± 1.7

[a] The Na^+,K^+-ATPase activities were measured immediately after elution of the protein from the BD-Talon beads. The protein was purified in either DDM or $C_{12}E_8$ with DOPS at the indicated concentrations.

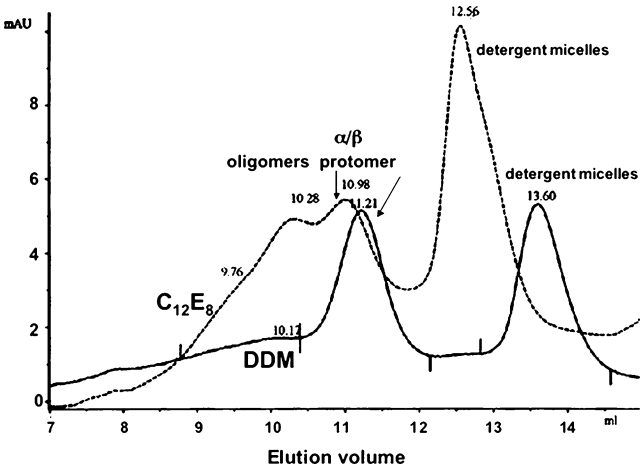

FIGURE 2: Size exclusion HPLC. Human α_1/porcine His_{10}*β_1 was prepared in $C_{12}E_8$/SOPS and concentrated to about 7 mg/L. Approximately 50 μg of the recombinant enzyme was injected into the running buffer containing either $C_{12}E_8$, 0.2 mg/mL, or DDM, 0.2 mg/mL, as indicated

$\beta)_n$, and detergent micelles. The all-porcine α_1/His_{10}*β_1 showed a similar pattern (not shown). For more dilute samples, the pattern was still polydisperse but the peak at ca. 11 min was more prominent (not shown). By contrast, when the protein prepared in $C_{12}E_8$ was separated in a running buffer containing DDM, it eluted mainly as the α/β protomer, in an almost monodisperse state. The identity of the peak at ca. 11 min, as the α/β protomer, was assigned by evaluating the apparent M_r using molecular mass standards to calibrate the column. The M_r was in the range 329000−343000 for the $C_{12}E_8$ and DDM samples. Taking into account values of bound detergent, which have been determined previously as 0.5−0.8 mg of $C_{12}E_8$ or DDM/mg of protein (*20*, *22*), this peak can only correspond to the α/β protomer.[3] As shown previously, the porcine α/β complex prepared in DDM, and separated by size-exclusion chromatography in DDM, also elutes almost exclusively as the α/β protomer, and analytical centrifugation also indicated that the renal Na^+,K^+-ATPase dissolved in DDM is the α/β protomer (*20*).

[3] Note that even without exact values for detergent binding the apparent mass of 329−343 kDa of the peak in DDM is too low to represent any oligomeric species other than the α/β monomer. For example, the protein mass of the $(\alpha/\beta)_2$ dimer alone, without bound detergent, is predicted to be 312 kDa, on the basis of that of α, 112 kDa, plus β, 44 kDa (*20*).

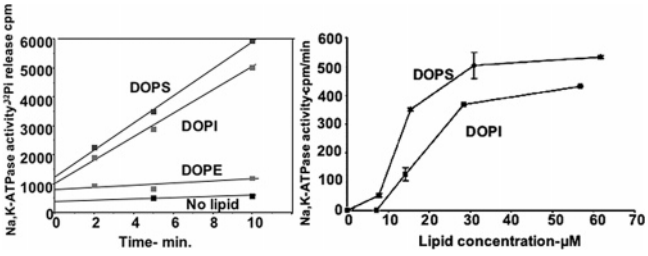

FIGURE 3: Na^+,K^+-ATPase activity with different phospholipids. Left: Porcine α_1/His_{10}*β_1 was prepared in $C_{12}E_8$, 0.1 mg/mL, with DOPS, DOPI, or DOPE, 0.05 mg/mL, or with no added lipid. Right: Porcine α_1/His_{10}*β_1 was prepared in $C_{12}E_8$, 0.1 mg/mL, and DOPS or DOPI, 0−0.05 mg/mL.

In the current experiments, DOPS was not added to the HPLC running buffer, but our previous work showed that the porcine α_1/β_1 eluted at the same time (ca. 11 min) when 0.02 mg/mL DOPS was added to the elution buffer containing 0.2 mg/mL DDM (*20*). Two important conclusions from the data in Figure 2 and Table 1 are (a) DDM easily dissociates oligomeric species of the protein present in $C_{12}E_8$ to the α/β protomer and (b) the minimal functional unit of Na^+,K^+-ATPase activity in either $C_{12}E_8$ or DDM is the α/β protomer. Specific Na^+,K^+-ATPase activity is not affected by the presence of higher oligomeric species present in $C_{12}E_8$.

Since oligomeric forms might be important for stabilization (*28*), we have looked at stability of the protein purified in either $C_{12}E_8$ or DDM, by incubating it for fixed times at 37 °C and then assaying Na^+,K^+-ATPase activity. The detergents were present at concentrations about 2 times the critical micelle concentration (with SOPS, 0.05 mg/mL), namely, in the standard elution conditions. The protein prepared with $C_{12}E_8$ was inactivated faster than that prepared with DDM (not shown), showing that the oligomeric species present in the $C_{12}E_8$-soluble protein did not protect against thermal inactivation. A number of other observations suggested that stability is affected primarily by the detergent−lipid competition and not by the presence of oligomers. For example, dilution of the protein prepared in DDM/DOPS (1:5 or 1:100) and incubation at 37 °C lead to progressively rapid loss of activity. Dilution cannot be associated with an increased proportion of oligomeric species, but on the other hand, it is associated with an increased ratio of detergent to protein. In addition, for preparations made with $C_{12}E_8$/DOPS, reducing the $C_{12}E_8$:DOPS ratio from 2:1 to 1:1 greatly reduced the rate of thermal inactivation at 37 °C (not shown). These observations suggest that detergent-mediated inactivation is caused by delipidation of the α_1/β_1 complex and point to specific lipid−protein interactions as the prime candidates for determining stability (see also ref *35*).

Stabilization of the α/β Complexes by Acid Phospholipids. The desired detergent/lipid composition can be readily modified during the washing steps and in the elution buffer of the BD-Talon metal affinity chromatography beads. Thus, the purification method described here allows convenient screening of lipid effects on Na^+,K^+-ATPase activity and stability. Qualitatively, stabilization by specific lipid effects was similar with either $C_{12}E_8$ or DDM, but some quantitative differences were observed.

Figure 3 presents an experiment with porcine α_1/β_1 complexes prepared in $C_{12}E_8$ showing that activity was supported by either acid phospholipid DOPS or DOPI, but

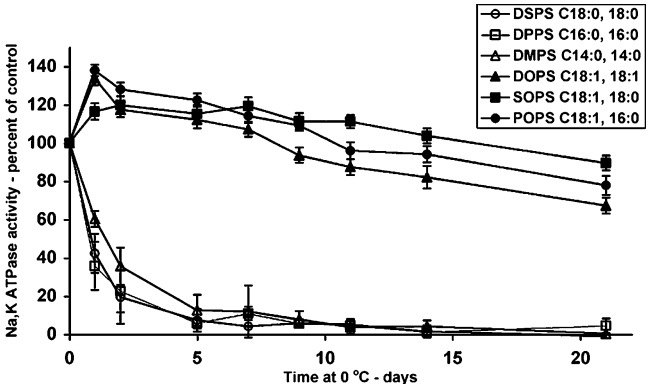

FIGURE 4: Na^+,K^+-ATPase activity and thermal stability with different PS variants. Porcine $\alpha_1/His_{10}*\beta_1$ was prepared in $C_{12}E_8$, 0.1 mg/mL, with DSPS, DPPS, DMPS, DOPS, SOPS, and POPS, 0.05 mg/mL, as indicated. The Na^+,K^+-ATPase activity was measured immediately after elution, and samples were then taken from the protein stored at 0 °C over 20 days for measurement of Na^+,K^+-ATPase activity at 37 °C.

somewhat better by DOPS. By contrast, the neutral phospholipid DOPE was barely able to maintain activity, and essentially no activity was observed without added phospholipid. Preparations made with mixtures of DOPS and DOPI with DOPE (0.025 mg/mL concentration of each lipid) showed the same activity as those made with DOPS or DOPI alone (not shown). Thus, DOPE does not compete with DOPS or DOPI, and the lack of activity observed with DOPE alone was presumably due to lack of correct binding to the protein. The neutral phospholipid DOPC sustains Na^+,K^+-ATPase activity, which was usually found to be similar to that seen with DOPS at 37 °C (see Table 3). However, DOPC stabilizes much less well than DOPS (see, for example, Figure 7 and also ref *20*).

Figures 4 and 5 and Table 2 show a comparison of the initial Na^+,K^+-ATPase activity and stability at 0 °C of the protein prepared with $C_{12}E_8$ and structural variants of phosphatidylserines, with different chain lengths and saturation at the *sn*-1 and *sn*-2 positions. As seen in Table 2, all six PS variants sustained Na^+,K^+-ATPase activity immediately after elution at 0 °C from the BD-Talon beads, with no significant difference among DOPS, SOPS, POPS, and DMPS and lower activities for DSPS and DPPS. Thus, there are no strict requirements for the chain length or saturation of the fatty acyl chain of the PS to observe the Na^+,K^+-ATPase activity per se. However, for all three PS variants with fully saturated side chains (C14, C16, or C18) Na^+,K^+-ATPase activity was rapidly lost over time at 0 °C (Figure 4). By contrast, Na^+,K^+-ATPase activity was preserved well at 0 °C only with PS variants having at least one unsaturated oleoyl chain in the *sn*-2 position. In addition, the Na^+,K^+-ATPase activity appeared to decline even more slowly with the asymmetric SOPS than with the symmetric DOPS (see Figure 6 for structures of DOPS, SOPS, and DSPS and also SLPS and DLPS used in the experiments). The latter observation was investigated more closely in Figure 5 for preparations made either with DOPS or with SOPS. Upon incubation at 37 °C (Figure 5, top) the Na^+,K^+-ATPase activity increased after a short incubation and then decreased over 1 h for both DOPS and SOPS, but the rate of inactivation was significantly slower with SOPS than with DOPS. Figure 5 (bottom) demonstrates an even more

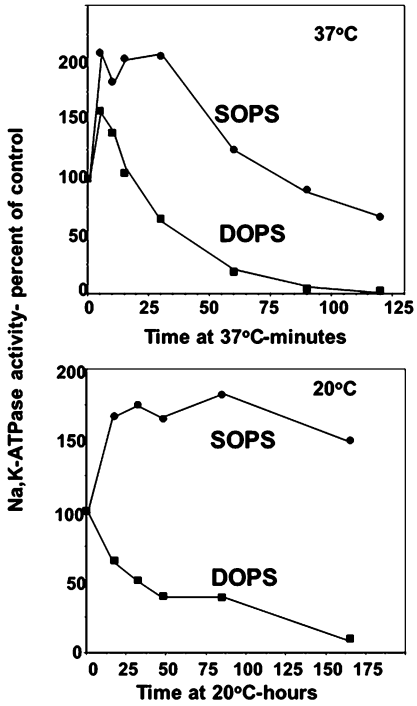

FIGURE 5: Comparison of thermal stability with either DOPS or SOPS. The porcine $\alpha_1/His_{10}*\beta_1$ complex was prepared in $C_{12}E_8$ and either SOPS or DOPS, 0.05 mg/mL. Samples were incubated for the indicated times at either 37 °C (top) or 20 °C (bottom), and Na^+,K^+-ATPase activity was then measured.

Table 2: Specific Na^+,K^+-ATPase Activity of Porcine α_1/β_1 Complexes Prepared with Different Phospholipids[a]

added lipid	Na^+,K^+-ATPase activity, (μmol/min)/mg ± SE	added lipid	Na^+,K^+-ATPase activity, (μmol/min)/mg ± SE
DOPS	11.4 ± 1.0	DPPS	6.5 ± 0.3
SOPS	11.9 ± 0.9	DSPS	4.7 ± 0.1
POPS	12.6 ± 1.2	none	1.7 ± 0.1
DMPS	11.5 ± 0.4		

[a] The Na^+,K^+-ATPase activities were measured immediately after elution of the protein from the BD-Talon beads. In all cases the protein was purified in $C_{12}E_8$, 0.1 mg/mL, with or without the indicated phospholipid, 0.05 mg/mL.

dramatic difference between DOPS and SOPS when the stabilities of the two preparations were compared at 20 °C. At this temperature, Na^+,K^+-ATPase activity of the preparation made with DOPS fell with a half-time of about 30 h, whereas that of the preparation made with SOPS rose initially and fell only very slowly over the next 165 h. The initial rise in the activity at both 37 and 20 °C may be indicative of equilibration of the protein with the lipid, prior to the thermal inactivation process. In many experiments, of which those in Figure 5 are typical, the peak activity was somewhat higher with SOPS than with DOPS, even though the initial activity was similar with the two PS variants. This reflects the better protection by SOPS against detergent-mediated inactivation over time.

Another experiment compared the activity and stability of preparations made with SOPS and two PS variants, either with the doubly unsaturated side chain, ω-6-linoleic acid, in both the *sn*-2 and *sn*-2 positions (DLPS-symmetric), or with stearic acid at *sn*-1 and linoleic acid at *sn*-2 (SLPS-asymmetric) (see Figure 6 for the structures). The results are presented in Figure 9 because they are relevant to the

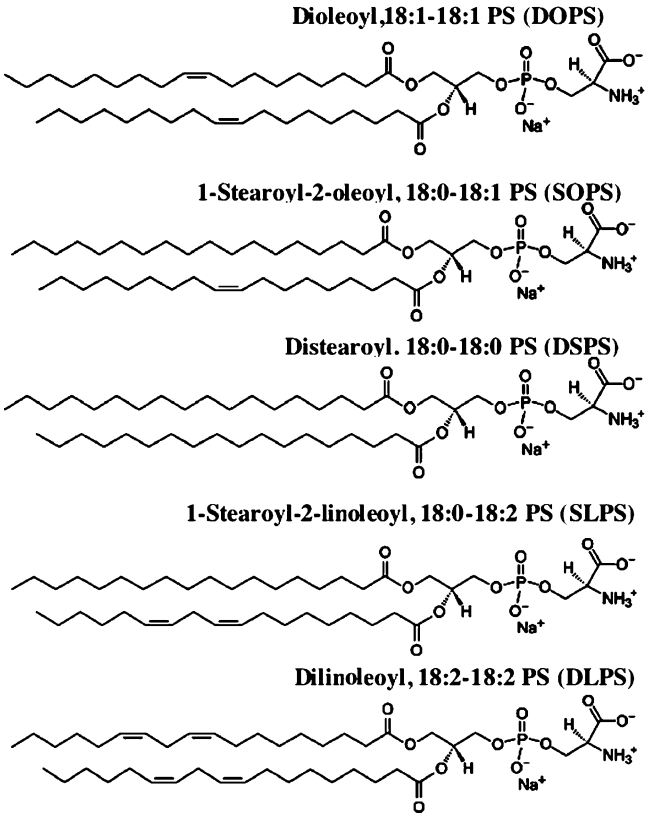

FIGURE 6: Structures of PS variants.

mechanism of action of cholesterol. In the preparations without cholesterol, the initial specific Na^+,K^+-ATPase activities were quite similar with all three PS derivatives. However, SOPS stabilized against thermal inactivation somewhat better than SLPS, and stabilization with the symmetric phosphatidylserine (DLPS) was much less effective than with either of the asymmetric phosphatidylserines.

Overall, the data in Figures 4, 5, and 9 show clearly that there is a strong selectivity for asymmetric PS structural variants for stabilization. By contrast, the structure of the PS derivative is less crucial when Na^+,K^+-ATPase activity is measured immediately upon elution from the beads (Table 2, Figure 9). For routine work, the asymmetric SOPS is now the PS of choice, rather than DOPS used previously, due to the improved stability and somewhat higher Na^+,K^+-ATPase activity.

Stabilization of the α/β Complexes by Cholesterol. Figures 7−10 and Table 3 show some striking stabilizing effects of cholesterol and evidence on its mechanism of action. The experiment in Figure 7 shows that the Na^+,K^+-ATPase activity of the human α_1/porcine β_1 complex is inactivated at 37 °C much more slowly with DOPS than with DOPC, as mentioned above, and also that the presence of cholesterol (0.01 mg/mL) together with either DOPS or DOPC confers strong additional protection against thermal inactivation. However, despite this strong stabilizing effect, an important observation with these and other different lipid combinations is that the initial Na^+,K^+-ATPase activity after elution from the beads was unaffected by the cholesterol (see Table 3). Cholesterol does not stabilize appreciably at 0 °C (not shown), presumably because the complex is sufficiently stable even without the cholesterol.

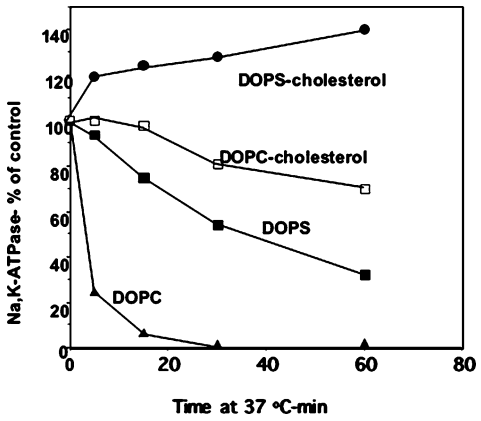

FIGURE 7: Thermal stability with either DOPC or DOPS, without or with cholesterol. The human α_1/porcine His_{10}*β_1 complex was prepared with $C_{12}E_8$ and either DOPS or DOPC, 0.05 mg/mL, without or with cholesterol, 0.01 mg/mL. The Na^+,K^+-ATPase activity was measured immediately after elution (Table 2, experiment 1). Samples were then incubated at 37 °C for the indicated times, and Na^+,K^+-ATPase activity was then measured.

When it became known that SOPS, itself, stabilizes even better than DOPS, it was of interest whether cholesterol would still provide additional stability (Figure 8). The experiment in Figure 8A shows that cholesterol had a very strong stabilizing effect, compared to SOPS alone, and essentially no inactivation was observed over 2 h at 37 °C for the preparation made with SOPS/cholesterol. In this experiment the maximal Na^+,K^+-ATPase activity was slightly raised in the presence of SOPS and cholesterol, while in other experiments the cholesterol did not affect the initial activity in the presence of SOPS (see also Figure 9). The Na^+,K^+-ATPase activity of the preparation made with cholesterol alone was low and was rapidly inactivated. The conclusion from Figure 8A is that the stabilizing effect of cholesterol requires the presence of the PS. Evidence on the mechanism of stabilization by cholesterol was obtained in the experiment in Figure 8B, which examined the dependence of activity on the concentration of SOPS, without and with cholesterol. The curve with cholesterol is significantly leftward shifted. In other words, cholesterol raised the "apparent binding affinity" of the SOPS (as well as raising the activity by about 20% in this experiment), implying the existence of an interaction between SOPS and cholesterol. SOPS and cholesterol could interact directly, or the effect of cholesterol could be indirect, exerted from a separate site on the protein. The experiment in Figure 9 provides some evidence on this issue. The activity and stability of preparations made with SOPS, DLPS, and SLPS, without and with cholesterol, were examined (see Figure 6 for the structures of SOPS, SLPS, and DLPS). Again, the initial activities were similar for all the PS derivatives, without and with cholesterol. As mentioned above, in the absence of cholesterol, the asymmetric PS derivatives (SOPS and SLPS) were more effective in protecting against thermal inactivation than the symmetric derivative (DLPS). The important observation, relating to cholesterol, is that the combination of cholesterol with either SOPS or SLPS protected fully, whereas the combination of cholesterol with DLPS protected only partially. It is known that cholesterol binds preferentially to saturated compared to monounsaturated, or especially polyunsaturated, fatty acyl

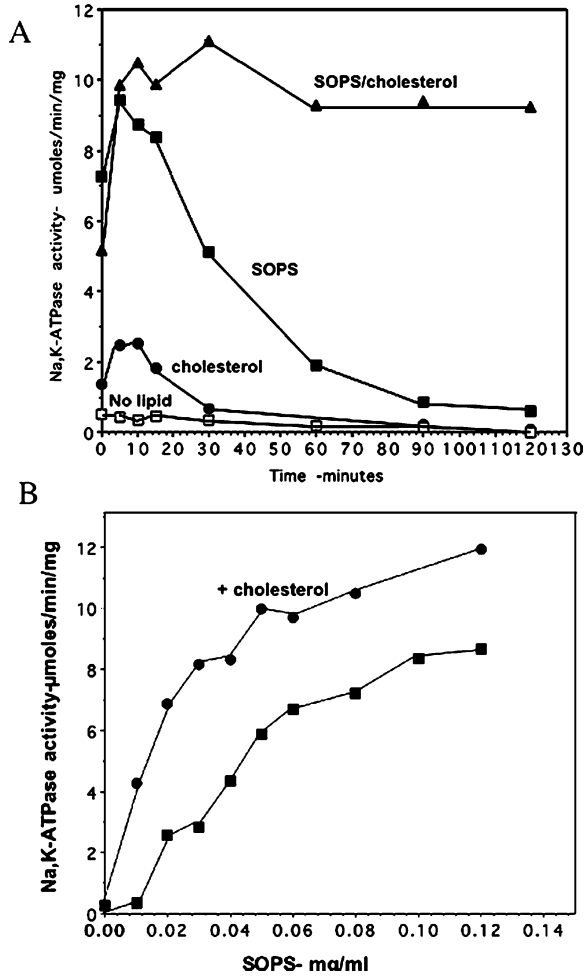

FIGURE 8: Combined stabilizing effects of SOPS and cholesterol. (A) The porcine α_1/His$_{10}$*β_1 complex was prepared in $C_{12}E_8$, 0.1 mg/mL, with SOPS, 0.05 mg/mL, alone, with SOPS, 0.05 mg/mL, and cholesterol, 0.01 mg/mL, with cholesterol, 0.01 mg/mL, alone, or without added lipids. The Na^+,K^+-ATPase activity was measured immediately after elution (Table 2, experiment 3). Samples were then incubated at 37 °C for the indicated times, and Na^+,K^+-ATPase activity was then measured. (B) The porcine α_1/His$_{10}$*β_1 complex was prepared in $C_{12}E_8$, 0.1 mg/mL, with SOPS, 0−0.12 mg/mL, without or with cholesterol, 0.01 mg/mL.

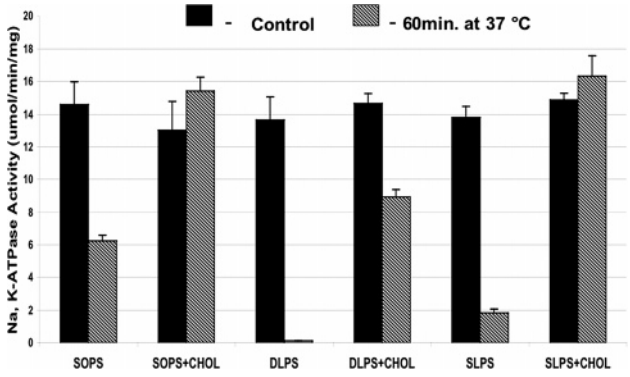

FIGURE 9: Comparison of Na^+,K^+-ATPase activity and stability of porcine α_1/His$_{10}$*β_1 complex prepared with SOPS, SLPS, and DLPS, without and with cholesterol. The porcine α_1/His$_{10}$*β_1 complex was prepared in $C_{12}E_8$, 0.1 mg/mL, with SOPS, DLPS, or SLPS, 0.05 mg/mL, without or with cholesterol, 0.01 mg/mL. The Na^+,K^+-ATPase activity was measured immediately after elution and after incubation of the preparations at 37 °C for 60 min.

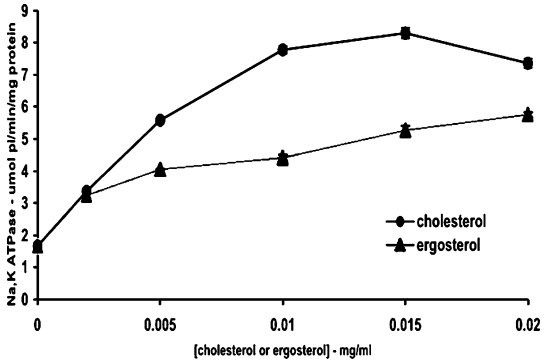

FIGURE 10: Comparison of the stabilizing effect of cholesterol and ergosterol. The porcine α_1/His$_{10}$*β_1 complex was prepared in $C_{12}E_8$, 0.1 mg/mL, with SOPS, 0.05 mg/mL, and either cholesterol or ergosterol, 0−0.02 mg/mL. The different protein samples were incubated for 60 min at 37 °C and samples then taken for assay of Na^+,K^+-ATPase activity.

chains (*36*, *37*). Thus, the implication is that the cholesterol and PS interact directly.

The experiment in Figure 10 compared the ability of cholesterol and the plant and yeast sterol, ergosterol, to protect against thermal inactivation. Preparations were made with $C_{12}E_8$/SOPS and varying concentrations of cholesterol or ergosterol. The preparations were warmed to 37 °C for 60 min, and Na^+,K^+-ATPase activity was assayed. Cholesterol protected strongly, and the standard concentration of 0.01 mg/mL (with 0.1 mg/mL $C_{12}E_8$/0.05 mg/mL SOPS) sufficed for a maximal effect. By contrast, ergosterol was much less effective than cholesterol. In other experiments, which examined the time course of inactivation, ergosterol was also much less effective in protecting the activity compared to cholesterol (not shown). At cholesterol concentrations higher than 0.02 mg/mL the Na^+,K^+-ATPase activity was inhibited.

In summary, cholesterol stabilizes the α/β complex strongly at 37 °C and probably interacts directly with the phosphatidylserine fatty acyl chain and the protein. Another important feature is that there is a clear distinction between the effects of the phospholipids and cholesterol on the stability of the α_1/β_1 complex and on the Na^+,K^+-ATPase activity per se (seen in Table 2 and Figure 9; see the Discussion for a possible explanation).

DISCUSSION

Expression and Purification of Functional Porcine α_1/β_1 Complexes. The new batch purification method described here for porcine α_1/β_1 and human α_1/porcine β_1 complexes (Figure 1) produces 80−90% pure Na^+,K^+-ATPase in one step. Due to its simplicity, and especially avoidance of an additional size-exclusion HPLC step, the new purification procedure represents a substantial improvement over the published method (*20*). Addition of low concentrations of EDTA to the DDM-solubilized membranes is important because it reduces binding of contaminant proteins to the BD-Talon beads. With this procedure it is possible to conveniently produce ca .1 mg of purified Na^+,K^+-ATPase from 1 g of membranes.

The specific Na^+,K^+-ATPase activity at 37 °C was essentially the same for the porcine α_1/β_1 and human α_1/porcine β_1 complexes, irrespective of the detergent used ($C_{12}E_8$ or DDM plus PS), in the range 8−16 (μmol of P_i released/min)/mg of protein. This specific activity is lower

Table 3: Specific Na^+,K^+-ATPase Activity of Human α_1/β_1 Complexes Prepared with Different Phospholipids, without or with Cholesterol[a]

added lipid	Na^+,K^+-ATPase activity, (μmol/min)/mg ± SE	added lipid	Na^+,K^+-ATPase activity, (μmol/min)/mg ± SE
DOPS	10.3 ± 0.6	DOPC	11.9 ± 1.4
DOPS/cholesterol	12.2 ± 1.0	DOPC/cholesterol	11.8 ± 0.3

[a] The Na^+,K^+-ATPase activities were measured immediately after elution of the protein from the BD-Talon beads. In all cases the protein was purified in $C_{12}E_8$, 0.1 mg/mL, with the indicated phospholipid 0.05 mg/mL, without or with cholesterol 0.01 mg/mL.

than the activities observed for purified membrane-bound renal Na^+,K^+-ATPase, 30−40 (μmol of P_i released/min)/mg (*32*). There are two possibilities to explain this difference. First, it cannot be excluded that a fraction of the protein is inactivated during the purification procedure. We continue to investigate this issue. Second, it is likely that a difference arises due to the change in the physical environment of the protein when removed from the lipid bilayer to the mixed protein−lipid−detergent micelles (in the membrane-bound state the molar activities of the recombinant and native renal Na^+,K^+-ATPase are similar (*20*)). Using recombinant Na^+,K^+-ATPase and pig kidney Na^+,K^+-ATPase labeled with FITC, we have recently observed $E_2(K)-E_1$ conformational transition of the recombinant protein and shown that the protein is strongly stabilized in the E_1 conformation by comparison with renal Na^+,K^+-ATPase (Belogus and Karlish, unpublished observations). A similar feature was observed previously for detergent-solubilized shark rectal gland Na^+,K^+-ATPase (*38*) and also for renal Na^+,K^+-ATPase reconstituted into soy bean lipid proteolipsomes (*39*). This feature could explain a lower molar activity.

Despite these detailed kinetic differences, it is important to note that the purified detergent-soluble recombinant Na^+,K^+-ATPase retains most of the fundamental characteristics of the membrane-bound renal Na^+,K^+-ATPase, including dependencies on Na, K, and ATP, partial reactions such as covalent phosphorylation, distinct E_1-E_2 conformational transitions, inhibition by ouabain with high affinity, and modulation by FXYD1 (*20*, *21*, Lifshitz et al., manuscript in preparation). Thus, there is good reason to believe that the active cation pumping mechanism is preserved intact.

Oligomeric Structure and Detergent Effects. The comparisons of the activity, oligomeric structure, and stability of the recombinant Na^+,K^+-ATPase prepared in either $C_{12}E_8$/PS or DDM/PS (Table 1, Figure 2) show clearly that the α_1/β_1 monomer is the minimal functional unit, and higher oligomeric species do not affect the activity. The conclusion on the minimal functional unit is similar to that reached previously in work with $C_{12}E_8$-solublized renal Na^+,K^+-ATPase (*22*, *29*, *40*) and our previous paper on the recombinant Na^+,K^+-ATPase (*20*). Interestingly, the two detergents are not passive bystanders but interact differently with the protein. DDM is a better solubilizer of the yeast membrane than $C_{12}E_8$ (*20*), it maintains the protein almost exclusively as the α_1/β_1 protomer, and it interferes strongly with interactions of FXYD proteins (*41*). However, DDM inactivates Na^+,K^+-ATPase less than $C_{12}E_8$. By contrast, $C_{12}E_8$ appears to be a "milder" detergent than DDM in that it solubilizes the yeast membrane inefficiently, it does not interfere with FXYD−α_1/β_1 interactions, and it does not interfere with subunit interactions, so that in $C_{12}E_8$ the protein consists of a polydisperse population of α_1/β_1 protomer and higher oligomers. However, $C_{12}E_8$ is a more potent inactivator of Na^+,K^+-ATPase than DDM, attributable to more effective delipidation of the protein (see also (*27*)). The polydisperse oligomeric species observed with $C_{12}E_8$ are easily dissociated into the α_1/β_1 protomer by low concentrations of DDM, and also by diluting the protein. Since, in addition, the Na^+,K^+-ATPase activity is not significantly different in the two detergents and the protein is less and not more stable in $C_{12}E_8$, compared to DDM, the implication is that the higher oligomers are neither well-defined nor stable, and thus represent nonspecific aggregates without defined roles in function or stabilization [suggested also previously (*29*)]. It is of interest that for another membrane protein, BmrA, size-exclusion HPLC has also shown a polydisperse pattern of protein in $C_{12}E_8$ but a monodisperse pattern in DDM (*42*). The fact that similar phenomena have been observed for two different membrane proteins, strengthens the conclusions on $C_{12}E_8$ as opposed to DDM discussed above, and suggests that they are general properties the two detergents.

Previously, size-exclusion HPLC coupled with low-angle laser light scattering photometry of renal Na^+,K^+-ATPase solubilized with $C_{12}E_8$ showed a distribution of oligomeric forms, which was affected by the ligand conditions and presence of PS (*22*−*24*). Although emphasis was placed on analysis of the individual oligomeric species, the conclusion was that the minimal functional unit is the α_1/β_1 protomer and the activity of $(\alpha_1/\beta_1)_2$ dimers is similar while that of $(\alpha_1/\beta_1)_4$ tetramers is about half. There does not seem to be a great discrepancy with the present results. Gel filtration of $C_{12}E_8$-solubilized shark rectal gland Na^+,K^+-ATPase showed that the protein can be eluted as an $(\alpha_1/\beta_1)_2$ dimer but, also, that raising the detergent to protein ratio dissociated the dimer, without loss of Na^+,K^+-ATPase activity (*27*, *28*). The dimer was much more thermally stable than the α_1/β_1 protomer. However, instability could have been caused by the delipidating effect of the higher $C_{12}E_8$ concentration required to dissociate the dimer, rather than by an intrinsic difference in stability of the $(\alpha_1/\beta_1)_2$ dimer and α_1/β_1 protomer. More recently, oligomers of DDM-solubilized α_1/β_1 subunits, expressed in insect cells, have been detected by coimmunoprecipitation assays (*43*). These might represent only a minor fraction of the pump units, since the major species of either the renal Na^+,K^+-ATPase or purified recombinant Na^+,K^+-ATPase in DDM is the α_1/β_1 protomer as determined by size-exclusion HPLC and ultracenrifugation (*20*). There is an extensive literature advocating the hypothesis that dimers or tetramers of the membrane-bound Na^+,K^+-ATPase α_1/β_1 protomer are required for function (reviewed in refs *30* and *31*). The evidence obtained here that the minimal functional unit of detergent-soluble Na^+,K^+-ATPase is the α_1/β_1 protomer indicates that higher order oligomers are not an essential requirement for Na^+,K^+-

ATPase function, although it does not exclude a role for oligomeric species in the intact membrane.

Stabilization by Specific Protein−Lipid Interactions. Previously, we inferred that DOPS binds specifically to and stabilizes the detergent-soluble purified Na^+,K^+-ATPase, measured as protection against detergent-mediated delipidation and thermal inactivation of Na^+,K^+-ATPase activity. Measurement of stabilization in this way is both convenient and supplies a sensitive measure of the requirements of a fully functional protein. The present findings, on stabilization by the different PS variants and cholesterol, strongly support the basic conclusion in ref *20* and provide insight into the mechanism of lipid−protein interactions. As discussed below, the stabilizing interaction may occur at the α/β subunit interface.

One basic assumption is that, in the absence of added lipid, the detergent inactivates the enzyme by displacing the endogenous yeast acid phospholipid. The phospholipid and fatty acid composition of *P. pastoris* membranes has been determined to be PA (7%), PS (6%), PE (15%), PC (50%), PI (7%), and CL (3−7%) and C16 (10%), C16:1 (5%), C18:0 (10%), and C18:1 (60−70%), respectively (G. Daum, University of Vienna, personal communication). Thus, there is a substantial amount of acid phospholipid (PS, PI, PA, and CL), and the majority is the dioleoyl variant, which should stabilize the expressed Na^+,K^+-ATPase similarly to DOPS, etc. It is of interest that quite similar specific Na^+,K^+-ATPase activities were observed initially with preparations made with the exogenous lipids DMPS, DOPC, DOPS, SOPS, and DOPS or SOPS or plus cholesterol, all of which have very different abilities to stabilize over time. Assuming that the added exogenous lipids replace the endogenous phospholipid during washes and elution from the beads, the implication is that Na^+,K^+-ATPase activity itself is not strongly dependent, if at all, on the structure of the exogenous lipid and on the specific lipid−protein interaction, which underlie the stabilizing effects. Thus, Na^+,K^+-ATPase activity is observed for some time even with suboptimal phospholipids before they are displaced by the detergent and inactivation takes place.

The selectivity for the acid phospholipid DOPS is not absolute since DOPI is also effective, but DOPI sustains a somewhat lower activity with a lower apparent affinity than DOPS. With the neutral phospholipid DOPC, although Na^+,K^+-ATPase activity is observed initially, it is rapidly inactivated. With DOPE, activity is largely inactivated during purification, presumably due to poor binding.[4] Overall, these stabilization experiments show strong selectivity for acid phospholipids and a limited selectivity for the head group, in the order serine (DOPS) > inositol (DOPI) > choline (DOPC) > ethanolamine (DOPE).

The most striking selectivity features of phospholipid structure observed include (*1*) a requirement for at least one unsaturated fatty acyl side chain at the *sn*-2 position and (*2*) optimal stabilization is achieved with the asymmetric SOPS compared to the symmetric DOPS, i.e., with stearoly at *sn*-1 and oleoyl at *sn*-2, a difference of a single double bond. The monounsaturated oleoyl side chain in SOPS also appears to be slightly preferable to the diunsaturated linoleic acyl chain in SLPS. The dependence of stabilization on chain length has not been looked at in detail, although the POPS variant with the C16 palmitoyl fatty acyl chain is not superior to the SOPS variant with C18 at the *sn*-1 position. A strong conclusion from the structural selectivity is that the PS interacts directly and specifically with the protein including, presumably, the serine head group and both fatty acyl chains. Phospholipids in mammalian membranes are usually asymmetric, with saturated and unsaturated fatty acyl chains at the *sn*-1 and *sn*-2 positions, respectively, and with an average fatty acyl chain length about C18 (*44*, *45*). The principal fatty acyl chains of PS of purified rabbit renal Na^+,K^+-ATPase are 16:0, (16%), 18:0 (27%), 18:1 (27%), and 18:2 (14%) (*46*). Thus, our findings are consistent with the assumption that stabilization by PS is a physiologically important function of this protein−lipid interaction and that the protein has evolved to interact optimally with the prevalent PS in the membrane.

Cholesterol strongly stabilized the detergent-soluble α_1/β_1 complex at 37 °C, but affected the initial specific Na^+,K^+-ATPase activity very little. The 10−20% higher specific Na^+,K^+-ATPase activity of many preparations prepared with cholesterol may also reflect protection against inactivation during purification. At 0 °C, cholesterol did not significantly stabilize the protein, perhaps because it is rather stable even without the cholesterol, but clearly inhibited Na^+,K^+-ATPase activity by about 50% (unpublished result). The following findings presented in Figures 7−10 suggest most simply that, at 37 °C, the phosphatidylserine, cholesterol, and α_1/β_1 complexes are involved in three-way specific stabilizing interactions: (a) The stabilizing effect of cholesterol at 37 °C is achieved only in the presence of PS. (b) Cholesterol raises the "apparent affinity" of SOPS. (c) Cholesterol is more effective at stabilizing in the presence of a PS with an unsaturated side chain at the *sn*-1 position (SOPS, SLPS) rather than a diunsaturated side chain (DLPS). Cholesterol is known to interact preferably with saturated rather than with monounsaturated and especially polyunsaturated fatty acid side chains and reduce the motional flexibility (*36*, *37*, *44*). (d) Cholesterol, the principal mammalian membrane sterol, is much more effective than ergosterol, the principal yeast membrane sterol. This selectivity is suggestive of a direct interaction with the protein.

Thus, overall, the data suggest that cholesterol binds both to the protein and to the saturated *sn*-1 fatty acid side chain of the SOPS (or SLPS), reducing its mobility and improving the interaction of the SOPS with the protein. On the basis of our findings, one could also hypothesize that one physiological role of cholesterol is to stabilize the α_1/β_1 complex at 37 °C, the physiological temperature. The molar ratio of cholesterol to SOPS required for a full stabilizing effect (0.01 mg/mL cholesterol:0.05 mg/mL SOPS) is ca. 40 mol %, a value within the physiological range of cholesterol:phospholipid ratios found in native membranes (*47*, *48*).

Comparison of Lipid Effects on Na^+,K^+-ATPase. Lipids as Solvents versus Specific Lipid−Protein Interactions. When comparing the present and previous observations on lipid requirements of the Na^+,K^+-ATPase, one must consider that

[4] A possible argument that added phospholipids stabilize the protein by binding the detergent and reducing its ability to inactivate would imply lack of selectivity in the phospholipid requirement and is excluded by this observation with DOPE (unless one assumes that different lipids bind detergent with variable affinity).

lipids can affect the activities of membrane proteins both by altering the physical environment of the protein and by more selective lipid−protein interactions (*25*, *26*). In native membranes, about 30 phospholipid molecules surround the Na^+,K^+-ATPase, and although these annular lipids are in rapid equilibrium with the bulk bilayer lipids, at any instant they constitute the immediate environment of the protein (*25*, *49*). The hydrophobic thickness, order, and packing of the annular lipids are thought to constrain the transmembrane helices in energetically favorable or unfavorable configurations and affect the conformational transitions, which underlie the function. Annular lipids have been seen in some high-resolution structures of membrane proteins (*50*, *51*). More often, however, X-ray structures of sufficient resolution have revealed individual lipid molecules nestling in crevices between transmembrane helices or at subunit interfaces. These are now referred to as "nonannular", "cofactor", or simply selectively bound phospholipids (*25*). Examples include a phosphatidylethanolamine molecule seen recently in a crystal structure of Ca^{2+}-ATPase (*7*) and a phospholipid molecule found at the subunit interface of the KcsA K^+ channel (*52*).

Early studies of phospholipid requirements of Na^+,K^+-ATPase showed that reactivation of delipidated enzyme (*53*, *54*) or reconstitution into proteolipsomes with defined lipids (*55*) was optimal in the presence of acid phospholipids, such as PS and PI, and also showed a dependence on the fatty acid chain length and saturation (*56*−*58*). By contrast, other reactivation studies utilized neutral phospholipids (PC and PE) and did not show a requirement for acid phospholipids (*59*). Also complete enzymatic hydrolysis of PS and PI in membrane-bound renal Na^+,K^+-ATPase only partially inactivated the Na^+,K^+-ATPase (*60*). The necessity to add acid phospholipids such as PS (or PI and PG) to detergent-solubilized renal Na^+,K^+-ATPase separated on HPLC columns (*22*, *24*), to maintain activity, is indicative of a stabilizing role. Nevertheless, these findings do not exclude the possibility of nonspecific electrostatic interactions between the acid phospholipid and the protein. By contrast, studies of lipid interactions with membrane-bound Na^+,K^+-ATPase by electron spin resonance (*49*) show that spin-labeled phosphatidylserine binds to the Na^+,K^+-ATPase, or a highly trypsinized preparation, relatively selectively, since the interaction is not affected by large changes in pH or salt concentrations (*61*, *62*). Binding of the negatively charged stearic or phosphatidic acid is less specific and is wholly or partially dominated by nonspecific electrostatic interactions.

Depletion and repletion of native membranes with cholesterol or reconstitution of proteoliposomes with phospholipids and cholesterol showed inhibitory (*48*, *57*, *63*) effects of cholesterol at high concentrations and activating effects at lower concentrations (*47*, *64*). In one study the Na^+,K^+-ATPase activity increased to a maximum as the molar ratio of cholesterol to phospholipid was increased from zero to the physiological ratio of about 0.4 and then fell as the ratio was increased further, suggesting a direct interaction with the protein (*47*, *65*). Cholesterol was also found to alter activation by cytoplasmic Na^+ ions as well as maximal rates (*63*, *64*) and also partial reactions such as phosphoenzyme formation (*64*, *66*). Also the effects of cholesterol were found to be selective in that other sterols such as lanosterol and ergosterol were much less effective (*47*, *67*). A more recent systematic study, using reconstituted proteoliposomes of defined lipid composition (*68*), found that the presence of cholesterol at 40 mol % decreases the optimal chain length of monounsaturated PC from 22C to 18C and increases the maximal rates of ATPase activity. Since cholesterol is known to increase the acyl chain order and bilayer thickness (*25*, *26*), these findings were interpreted to mean that the hydrophobic thickness of the bilayer and matching with the protein are critical features, as well as lipid order. In a second study the cholesterol was found to affect several partial reactions of the Na^+,K^+-ATPase cycle, including phosphorylation and conformational transitions, but not all effects could be explained by the membrane-thickening effect of cholesterol (*69*). In another study the molar activity of Na^+,K^+-ATPase in membranes from rat and toad kidney and brain was correlated strongly with the average molecular area per membrane lipid molecule, which was progressively reduced at higher cholesterol:phospholipid ratios (*70*).

In summary, many of the functional effects of phospholipids and cholesterol on Na^+,K^+-ATPase activity, detected in native or artificial bilayers, are attributable to solvent effects, in particular to hydrophobic matching and lipid order and packing. Previous observations might also have included stabilizing interactions of acid phospholipids and cholesterol, which went unrecognized. Cholesterol has not been considered previously as a stabilizing agent, although our results show that the stabilizing effect is very strong. Indeed, the selective effects of phosphatidylserine variants (e.g., SOPS) and cholesterol, detected with the detergent-solubilized recombinant protein, are primarily stabilizing interactions, due to specific interactions, which do not greatly affect the molar Na^+,K^+-ATPase activity.

Do Lipids Interact near the α/β Subunit Interface? Previously, we proposed that the protection by DOPS against inactivation of Na^+,K^+-ATPase activity both in a K^+-rich medium and after deglycosylation of the β subunit is explained simply by assuming that the PS binds at the interface of α and β subunits and stabilizes their interaction (*20*). Since α and β subunit interactions are known to play a major role in stabilizing the α subunit (*10*), the observation that SOPS and cholesterol primarily stabilize the α_1/β_1 complex, and do not affect Na^+,K^+-ATPase activity, is compatible with a role in α and β subunit interactions. On the basis of the preference for an acid phospholipid, and selectivity for the fatty acyl chains of PS, presented here, one could infer both that the negatively charged head group interacts with positively charged side chains at the membrane surface of α or β subunits and that the *sn*-1 and *sn*-2 fatty acyl chains interact with transmembrane segments. The head group of the PS and interacting side chain are likely to be located at the cytoplasmic face since PS is normally oriented heavily toward the cytoplasmic surface in native membranes (*71*). The selective effects of cholesterol imply that it binds to the protein and interacts with a fatty acyl chain of PS and stabilizes its interaction with the protein. In a forthcoming paper, which describes purification and stabilization of α_1 and α_2 isoforms, we shall provide further evidence that the PS and cholesterol interact with M8, M9, or M10 of α (Lifshitz et al., manuscript in preparation). Another suggestive observation is that Na^+ ions selectively stabilize the $\alpha_1\beta_1$ complex by comparison with K^+, Rb^+, choline, and other cations (H. Haviv, unpublished observations). Selectivity for

Na^+ ions implies most simply that the third Na^+ occlusion site is involved. The third Na^+ site is thought to be located within M9, and M8 and M6 of α (*72*), near the α/*β* subunit interface and the proposed surface of interaction with the lipid.

CONCLUSION

The optimization of lipid−protein interactions, described here, has led to a preparation of purified, functional, and stable recombinant Na^+,K^+-ATPase in 1−2 mg quantities. The quantity and quality of this protein are sufficient for initiation of crystallization trials and a variety of detailed biochemical and biophysical studies.

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