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Purification of the Human α2 Isoform of Na,K-ATPase Expressed in *Pichia pastoris.* Stabilization by Lipids and FXYD1†

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ABSTRACT: Human α1 and α2 isoforms of Na,K-ATPase have been expressed with porcine 10*Histidine-tagged β1 subunit in *Pichia pastoris*. Methanol-induced expression of α2 is optimal at 20 °C, whereas at 25 °C, which is optimal for expression of α1, α2 is not expressed. Detergent-soluble α2β1 and α1β1 complexes have been purified in a stable and functional state. α2β1 shows a somewhat lower Na,K-ATPase activity and higher $K_{0.5}$K compared to α1β1, while values of $K_{0.5}$Na and K_mATP are similar. Ouabain inhibits both α1β1 ($K_{0.5}$ 24.6 ± 6 nM) and α2β1 ($K_{0.5}$ 102 ± 14 nM) with high affinity. A striking difference between the isoforms is that α2β1 is unstable. Both α1β1 and α2β1 complexes, prepared in $C_{12}E_8$ with an added phosphatidyl serine, are active, but α2β1 is rapidly inactivated at 0 °C. Addition of low concentrations of cholesterol with 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (SOPS) stabilizes strongly, maintaining α2β1 active up to two weeks at 0 °C. By contrast, α1β1 is stable at 0 °C without added cholesterol. Both α1β1 and α2β1 complexes are stabilized by cholesterol at 37 °C. Human FXYD1 spontaneously associates in vitro with either α1β1 or α2β1, to form α1β1/FXYD1 and α2β1/FXYD1 complexes. The reconstituted FXYD1 protects both α1β1 and α2β1 very strongly against thermal inactivation. Instability of α2 is attributable to suboptimal phophatidylserine−protein interactions. Residues within TM8, TM9 and TM10, near the αβ subunit interface, may play an important role in differential interactions of lipid with α1 and α2, and affect isoform stability. Possible physiological implications of isoform interactions with phospholipids and FXYD1 are discussed.

Na,K-ATPase actively transports Na and K ions across cell membranes and maintains the normal electrochemical gradients of the cations, which regulate fluid and electrolyte balance. The Na,K-ATPase is also the receptor for cardiac glycosides which have been used for over two hundred years to treat heart failure. More recently a signal transducing function, which is triggered by binding of cardiac glycosides and modulates cell growth, apoptosis and cell adhesion, has been described (*1*, *2*).

The Na,K-ATPase consists of two subunits, α and β, in an αβ heterodimeric complex. α is the catalytic subunit, and contains the sites for ATP, Na, K, and Mg ions, and also the sites for cardiac glycosides (*3*−*5*). β is required for stabilization and transport of α to the cell membrane (*6*), and there is also evidence for involvement of the β subunit in cell−cell adhesion and polarized expression of the pump in epithelial cells (*7*). The kinetic properties of the αβ heterodimer are modulated by association with FXYD proteins, which are expressed in a tissue-specific fashion (*8*−*10*). There are four known isoforms of the α subunit (α1, α2, α3, and α4), which show about 85% sequence similarity, and three known isoforms of the β subunit (β1, β2, and β3), which show about 43% sequence similarity (*11*). The isoforms show a tissue-specific and developmentally regulated pattern of expression. α1 is found in nearly all animal tissues and is the "housekeeping" isoform. α2 is expressed in heart, skeletal and smooth muscle, brain (mainly astrocytes), adipocytes, eye and lung. α3 is found primarily in brain (mainly neurons), but also in human heart. α4 is expressed in sperm. The β1 isoform is expressed ubiquitously, β2 is found primarily in muscle and brain and β3 in a variety of tissues. α1, α2, α3 and β1 isoforms are expressed in human heart (*12*, *13*).

α2 is of particular interest, as has become clear from a long series of studies comparing $\alpha1^{\pm}$ -and $\alpha2^{\pm}$-haploinsufficient mice (full knock-out mice $\alpha1^{-/-}$ and $\alpha2^{-/-}$ are not viable), and effects of ouabain on mice with α2 or α1 subunits engineered to be insensitive or sensitive to ouabain, respectively (*14*). Initial studies showed that both α1 and α2 appeared to play different roles for cardiac and skeletal muscle contraction (*15*, *16*). Subsequent studies showed that both α1 and α2 isoforms can mediate positive inotropy and hypertension, induced by cardiac glycosides, but α2 seems to plays a predominant role, even though α2 is expressed at much lower levels than α1 (*17*−*22*). These studies also showed that the cardiac glycoside binding site plays a role in blood pressure regulation, presumably by binding endogenous mammalian cardiac glycoside (*20*, *23*, *24*). In smooth muscle and astrocytes α2 is confined to microdomains on the cell surface, in proximity with NCX1, and overlying the sarcoplasmic reticulum, by contrast with α1, which is more

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ubiquitously distributed (*25*−*29*). In cardiac myocytes, α2 is more densely located in T-tubules than in the external sarcolemma, by contrast with α1, which is more evenly distributed, and α2 carries most of the pump current in the T-tubules proximal to the sarcoplasmic reticulum (*30*−*32*). It is thought that the reticulate distribution of α2, and proximity to the other proteins involved in Ca signaling, allows efficient participation of α2 in Ca signaling within the sarcoplasmic (endoplasmic) reticulum, without alteration of bulk cytoplasmic Na and Ca concentrations (*22*, *33*−*35*). An unrelated but significant finding concerning human α2 is that a number of missense mutations are involved in familial hemiplegic migraine (FHM[1]), a rare but severe autosomal dominant subtype of migraine with aura. The majority of FHM families have a mutation in a voltage-sensitive Ca channel, but about 25% of FHM cases (type 2) are attributed to mutations to α2, which cause alterations in active Na and K transport (*36*, *37*).

Isoforms of the α subunit have been expressed in various cells and the enzymatic properties studied [reviewed (*11*)]. Human α1, α2, and α3 isoforms have been expressed in *Xenopus oocytes*, together with β1, β2 or β3 isoforms, producing nine combinations in all (*38*). In the yeast *Saccharomyces cerevisiae* α1, α2 and α3 have been expressed with β1, but α2 was expressed at low levels, due apparently to instability (*39*). The transport and ouabain binding properties of the recombinant proteins have been studied in some detail. For example in *Xenopus oocytes*, the turnover rates are lower and $K_{0.5}$ K activation are somewhat higher for α2 compared to α1 (*38*). All three human isoforms bind ouabain with a high affinity (*38*−*40*), but α2 shows much faster rates of association and dissociation than either α1 or α3 (*38*). We have reported functional expression of both porcine and human α1 with porcine β1 in the methanotrophic yeast *Pichia pastoris* (*41*−*43*), and a preliminary statement on expression of human α2 with porcine β1 (*44*). Human α3 and β1 have also been expressed in *P. pastoris* (*45*).

Recently, we have described purification of detergent-soluble porcine α1β1 and human α1 porcine β1 complexes expressed in *P. pastoris* (*42*, *43*). In the most recent development 80−90% pure and active α1β1 complexes, in either DDM or $C_{12}E_8$, have been obtained in a single step (*43*). A crucial feature was the necessity to add phosphatidyl serines (PS) in order to maintain stability of the proteins, which was undermined by detergent-mediated delipidation. Most recently, selectivity studies, using different phosphatidyl serines, showed that stability was maintained best by SOPS an asymmetric phosphatidyl serine with stearoyl and oleoyl fatty acid at sn1 and sn2 positions, respectively. Strikingly also, low concentrations of cholesterol conferred strong additional stabilization in the presence of SOPS, when examined at 37 °C. At 0 °C the α1β1 complexes are stable even without cholesterol. The PS and cholesterol were proposed to bind specifically to the protein, and to each other, near the αβ subunit interface, and stabilize the subunit interaction (*42*, *43*). Approximately 1−2 mg quantities of the purified and stable α1β1 complexes can be prepared conveniently, making these preparations suitable for crystallization trials and detailed functional and other biochemical and biophysical studies.

Purification of the α2 isoform, which is the objective of this paper, has not been achieved previously, and could be important for detailed study of α2-specific functional properties, pharmacological profiles with cardiac glycosides and interactions with other proteins. Because the human α1 porcine β1 complex has been purified in a stable and active form, it seemed that purification of human α2 porcine β1 complex might be readily achieved. However, it quickly became clear that α2 is unstable, as suggested also by low expression in *S. cerevisiae* (*39*). Thus, the major challenge has been to understand the reason for instability, and utilize the knowledge to stabilize the protein. Interactions with lipids (PS and cholesterol) have been found to be the key factor in determining stability of α2, as found previously with α1 (*42*, *43*), although α2 shows qualitatively different lipid−protein interactions. Another important route to stabilizing the protein involves specific interactions with FXYD1 (*9*, *10*). FXYD1 is the principal FXYD protein expressed in heart and skeletal muscle, and is the substrate for both PKA and PKC phosphorylation. There is much current interest in functional effects of FXYD1 and effects of phosphorylation, in a variety of experimental systems. An initial publication from this laboratory described functional effects of FXYD1 on the purified porcine α1β1 complex purified from *P. pastoris* (*46*). The present work describes a phenomenon of stabilization by FXYD1 and, in particular, focuses on the question whether both human α1 and α2 isoforms are stabilized by FXYD1. Possible isoform selectivity was of interest, because there are some discrepancies between different studies as to whether FXYD1 regulates α2 as well as α1 (*30*, *47*). The result of this work is that a purified, active and stable, preparation of α2β1 has been obtained, and also new insights into interactions of α1 and α2 isoforms with lipids and FXYD1, and the relations between these phenomena.

EXPERIMENTAL PROCEDURES

Yeast Media. YPD: 1% Bacto yeast extract, 2% bactopeptone, 2% dextrose, to solidify the medium 2% Bactoagar were 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.

Construction of Plasmids for the Expression of Human α1 or α2 with Porcine β1, and Expression of Human FXYD1. A pHIL-D2 vector (Invitrogen) construct containing cDNAs encoding porcine α1 (accession X03938) and porcine β1 (accession no. X04635) with a 10xhistidine tag at its N′ terminus (α1/His_{10}*β1) has been described previously (*42*). Human α1 (accession X04297) in vector pNKS2 and human α2 (accession NM_000702) in vector pSD5 were a gift from K. Geering, Univ. Lausanne, Switzerland. The coding regions

[1] Abbreviations: FHM, familial hemiplegic migraine; 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-trinitriloacetic acid; EDTA, ethylene diamine tetraacetic acid; PS, phosphatidyl serine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]; cmc, critical micelle concentration.

were excised with *Xba*I and subcloned into the pHIL-D2 plasmid (α1/His_{10}*β1) thus replacing the porcine α1 insert by the human orthologues and producing the constructs pHIL-D2(human α1/porcine His_{10}-β1) and pHIL-D2(human α2/porcine His_{10}-β1), respectively. Proper insertion and orientation has been verified by sequencing. Human FXYD1 (accession H23593) was subcloned into the pHIL-D2 vector

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 (human α1/porcine His_{10}*β1 or human α2/porcine His_{10}*β1) constructs. Preparation of spheroplasts and selection for His^+, Mut^s transformants and dot blot analysis, to screen for maximal copy number, was done according to the Invitrogen manual (Version F) and described previously (*41*, *42*). Following transformation, approximately 200−300 His^+ prototrophs were replica plated on MM (1.34% YNB, 4 × 10^5% biotin, 0.5% methanol, 2% agar) and MD (1.34% YNB, 4 × 10^5% biotin, 2% dextrose, 2% agar) plates for screening of Mut^s (methanol utilization slow) clones.

Candidate Mut^s clones were inoculated in 5 mL of fresh BMG broth, with vigorous shaking at 25 °C (α1) or 20 °C (α2), 0.5% methanol was added daily for 5 days. Cells were pelleted and membranes prepared as described below to identify the clones expressing the highest protein level of human α1, or α2. Standard 3 L cell cultures were grown in a Bellco spinner flask, with methanol induction of Na^+,K^+-ATPase expression, as described previously (*42*), using a controlled temperature of either 25 °C (α1) or 20 °C (α2), respectively. The cultures reached an OD600 of ca. 25., representing about 75 g/3l.

The pHIL-D2 vector containing human FXYD1 was used to transform spheroplasts of SMD1165 and His4 and Mut^s clones were selected. Dot blot analysis was done to scan for optimal DNA copy number integration of FXYD1 (as described in 46). *P. pastoris* clones maximally expressing human FXYD1 (without αβ) were used for *in vitro* reconstitution experiments.

Membrane Preparations. P. pastoris cells were broken with glass beads and urea-treated membranes were prepared as described in (*42*). Roughly 1 g of membrane protein was obtained per 100 g of cells. Pig kidney Na^+,K^+-ATPase was prepared as described in (*48*).

Purification of α1β1 and α2β1 Complexes (43). Membranes expressing human α1/porcine His_{10}*β1 or human α2/porcine His_{10}*β1 were homogenized at 0 °C for 5 min with DDM, at a ratio of DDM:protein of 2:1 w/w, in a medium containing NaCl, 250 mM; Tricine-HCl, 20 mM pH 7.4; imidazole, 5 mM; PMSF, 0.5 mM; and glycerol, 10%. The DDM was at 2 mg/mL and protein 1 mg/mL. The unsolubilized material was removed by ultracentrifugation. EDTA was added at 50 μM. The DDM-solubilized membranes were then incubated overnight at 4 °C with BD Talon (Co^{2+}-chelate) beads, at a ratio of 100 μL beads per supernatant from 10 mg of membranes. The beads were washed twice for 5 min at 0 °C with gentle mixing and aspiration of five equivalent volumes of a solution containing NaCl, 100 mM; Tricine·HCl, 20 mM pH 7.4; $C_{12}E_8$, 0.1 mg/mL; DOPS or SOPS 0.05 mg/mL or other lipids and cholesterol at 0.01 mg/mL, as indicated; glycerol, 25%, and imidazole, 10 mM. Protein was eluted by mixing the beads for 60 min at 0 °C with one equivalent volume of the same washing buffer containing imidazole, 150 mM. The eluted protein was stored at 0 °C. 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 DDM 2 mg/mL.

The protein concentration of the α1/β1 and α2/β1 complexes was determined by comparing Coomassie stain of the α subunit with that of a standard amount of pig kidney Na^+,K^+-ATPase. We have shown recently that this method overestimates the true protein concentration by 2-fold due to a difference in the purity of the test (recombinant ca. 80% pure) and standard (renal ca. 40% pure) (*43*). Specific activities are calculated on the basis of the correct protein concentration.

In Vitro Reconstitution of α1β1/FXYD1 and α2β1/FXYD1 Complexes. Membranes expressing human FXYD1 were solubilized at 0 °C for 5 min in DDM (DDM:protein, 2:1 w/w), and concentrated by ultrafiltration (Centriprep YM-30 cat. No. 4322 by Amicon, Millipore) at 1500*g* for 2 h at 4 °C. The retentate fraction containing FXYD1 in micelles was then incubated overnight at 4 °C with BD-Talon beads prebound with the α1β1 or α2β1 complex, as described (*46*). The αβ/FXYD1 complexes formed was then eluted as described above.

SDS−PAGE, Immunoblots, Size Exclusion HPLC. 2−5 μg of recombinant enzyme or 40 μg of yeast membranes was separated on 7.5%, or 10% polyacrylamide SDS−Tricine gels (*49*). Gels were stained with Coomassie, scanned with an imaging densitometer (GS-690, BioRad) and analyzed using the Multi-analyst software (BioRad). Immunoblots were blotted with anti-KETYY antibody that recognizes the C-terminus of the α subunit, or with anti-β antibodies raised against the extracellular domain of the β subunit (*50*). FXYD1 was detected with an anti-FXYD1 antibody raised against the C-terminal sequence CRSSIRRLSTRRR (*46*).

Size exclusion HPLC was done using a Superdex 200 column (300 × 10 mm) (Amersham Pharmacia Biotech) and Ettan LC chromatography system. 50−100 μg of purified recombinant Na,K-ATPase complexes was injected onto the column in a running medium containing NaCl 150 mM, HEPES 50 mM pH 7.4, $C_{12}E_8$ 0.1 mg/mL The protein was eluted at 0.5 mL/min, in a medium containing NaCl, 150 mM; Na^+-HEPES, 50 mM pH 7.4; $C_{12}E_8$ 0.1 mg/mL (*42*, *43*).

Na^+,K^+-ATPase Assays. Measurement of Thermal Stability. Na^+,K^+-ATPase assays at 37 °C on the purified enzyme were performed as described in (*41*−*43*). 1 μL of recombinant enzyme (≈0.2−0.6μg of protein) was added to 100 μL of the standard reaction medium containing 1 mM ATP+ [γ-^{32}P-ATP]; NaCl, 130 mM; KCl, 20 mM; $MgCl_2$, 3 mM; Histidine, 30 mM pH 7.4; EGTA, 1 mM. For measurement of kinetic parameters the concentrations of ATP, Na or K were varied at constant ionic strength balanced with choline chloride. For measurement of $K_{0.5}$ Na the Na concentration was varied with 150 mM (Na + choline) and 100 mM KCl. Some experiments were done with a colorimetric assay using malachite green dye to detect the phosphomolybdate (P_i Color Lock, Innova Biosciences). ^{32}P-release (or P_i release in the colorimetric assay) was usually measured in duplicate at three time points over 10−15 min. The rate of Na^+,K^+-ATPase activity was calculated from the linear slope ± SE (range of SE 1−10%) (see for example Figure 4).

Rates of ATP hydrolysis at different Na^+ or K^+ (X) concentrations were fitted to the function $v = V_{max}[X]^{nH}/([X]^{nH} + K_{0.5}^{nH})$. $K_{0.5}$ is the concentration of cation required for half-maximal stimulation of ATP hydrolysis and nH is the Hill coefficient. The function $v = V_{max}[ATP]/([ATP] + K_m)$ was used to fit curves at different ATP concentrations. The data for individual experiments was fitted to the functions, and values of v/V_{max} were calculated. Then values of v/V_{max} for replicate experiments were averaged for each Na^+, K^+, or ATP concentration, and the best-fit average parameters $K_{0.5} \pm$ SE, nH $\pm$ SE or $K_m \pm$ SE were recalculated.

Inhibition by ouabain was evaluated by adding the inhibitor to the reaction medium just prior to addition of protein. ATP hydrolysis was measured at 37 °C over 60 min. Values of the rates ± SE were used to calculate the ratio v/v_0 at each ouabain concentration. Average values of v/v_0 for replicate experiments were calculated and $K_{0.5} \pm$ SE was obtained by fitting the data to $v/v_0 = K_{0.5}/([ouabain] + K_{0.5})$. Kaleidagraph version 3.51 (Synergy Software) was used for fitting all experimental data to kinetic functions.

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. In some thermal inactivation experiments the samples were assayed in duplicate for one time (10 or 15 min), with values differing by less than ten percent. For these experiments error bars do not appear (e.g Figures 6 and 9).

Ouabain binding to yeast membranes using [^{3}H-ouabain] were done in triplicate in the conditions described in ref (*51*). The binding was done at a single concentration of 400 nM ouabain, which was assumed to be saturating, in order to measure the maximal binding capacity.

Materials. Escherichia coli DH5a was used for propagation and preparation of various plasmid constructs. *P. pastoris* strain SMD1165 (his4, prb1) was used for transformation. Yeast Lytic Enzyme ICN Biomedicals Inc, cat. No. 152270 was used for DNA dot blots. BD Talon metal affinity resin (cat. No. 635503) was purchased from Clontech; *n*-dodecyl-β-D-maltopyranoside, ANAGRADE (cat. No. D310); octaethylene glycol monododecyl ether, ANAGRADE ($C_{12}E_8$) (25% W/W) (cat. No. O330) were from Anatrace. Synthetic lipids: 1,2-dioleoyl-*sn*-glycero-3-(phospho-L-derine) (sodium salt) (DOPS cat. No. 830035); 1-stearoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine) (sodium salt) (SOPS cat. No. 840039); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, cat. No. 850375), were obtained from Avanti and stored as chloroform solutions. Cholesterol was purchased from Sigma. [^{32}P]-ATP and [^{3}H]-ouabain were from Amersham. FXYD1 ultrafiltration was done by Centriprep YM-30 cat. No. 4322 obtained from Amicon, Millipore. All other materials were of analytical grade.

RESULTS

Expression and Purification of $\alpha 2\beta 1$. The human α1 and α2 isoform cDNA's were cloned into the pHIL-D2 vector containing the pig His10-β1 cDNA, and the resulting vectors, containing human α1 porcine β1 ($\alpha 1\beta 1$) or human α2 porcine β1 ($\alpha 2\beta 1$), under the AOX1 promoter, were used to transform the SMD1165 protease-deficient strain of *P. pastoris*. His_4 and Mut^s strains were isolated, as described previously (*41*−*43*). Initially Mut^s clones were grown at 25 °C, a temperature optimal for expression of porcine α1 (*41*). At 25 °C, human α1 showed high expression as found for porcine α1, but human α2 showed no expression (Figure 1A, lane 3). Previously, unstable mutants of the α1 subunit have been expressed at reduced temperatures (*52*). Since human α2 is expressed in *S. cerevisiae* at low levels and is unstable (*39*), Mut^s clones expressing α2 were also grown at 15 °C and 20 °C. Indeed, at 20 °C, some expression of α2 was observed, and less at 15 °C (Figure 1A, lanes 1 and

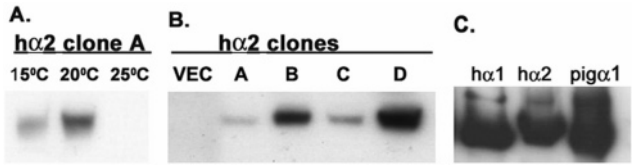

FIGURE 1: Expression of α2 in *P. pastoris* at 20 °C. Detection of the α subunit expressed in membranes with immunoblots probed using an antibody against the C-terminal residues of α1 and α2 (anti-KETYY). (A) α2 clone grown at 15, 20, and 25 °C, respectively. (B) Separate Mut^s clones grown at 20 °C. (C) Comparison of expression of human α1 and α2 and porcine α1 grown in 3 L spinner bottles.

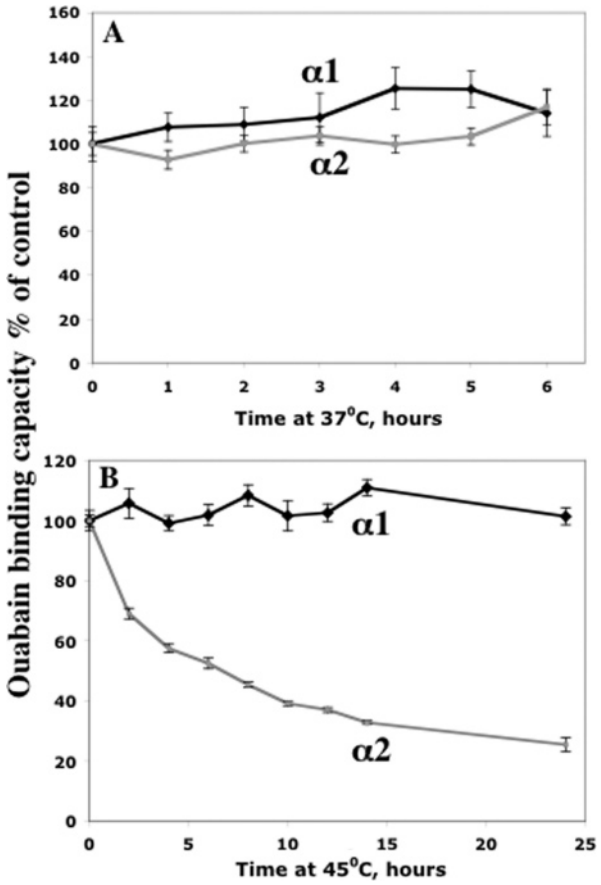

FIGURE 2: Thermal stability of ouabain binding to α1 and α2 in *P. pastoris* membranes. Membranes expressing α1 or α2 were incubated for the indicated times at either 37 °C (A) or 45 °C (B), and then ^{3}H-ouabain binding was measured. The maximal capacities for ouabain binding, measured at 400 nM ouabain, were 19 pmol/mg for α1 and 15 pmol/mg for α2.

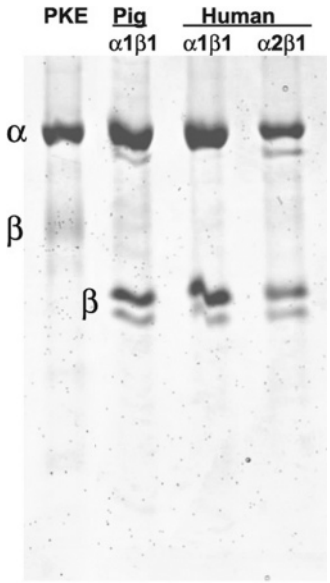

FIGURE 3: Purification of α1β1 and α2β1 complexes. Coomassie stained gels of porcine α1β1, human α1 porcine β1, or human α2 porcine β1 complexes. PKE, pig kidney Na,K-ATPase.

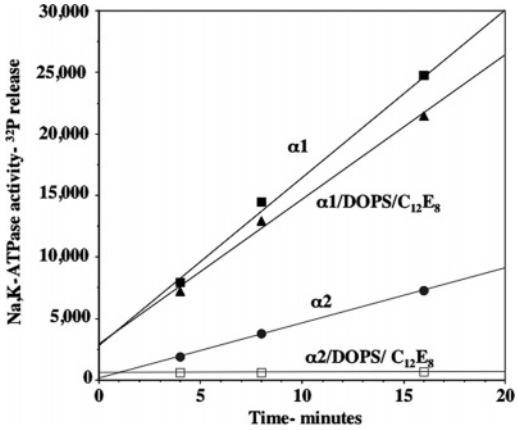

FIGURE 4: Time-course of Na,K-ATPase activity of α1β1 and α2β1 complexes without and with added DOPS/$C_{12}E_8$. ^{32}P release was measured over 15 min without or with added $C_{12}E_8$, 0.1 mg/mL plus DOPS 0.05 mg/mL in the reaction medium, as indicated (see Experimental Procedures).

2). Other experiments showed that maximal expression was achieved already on the first day of expression following methanol induction. Further screening (Figure 1B) of Mut^s transformants, grown at 20 °C, revealed clones, which expressed human α2 at much higher levels (compare clones B and D with clone A). Clones B and D were grown in small volumes and Na,K-ATPase activity and specific ouabain binding were measured on membranes (data not shown). The specific ouabain binding for α2-clones, B and D, was 15.24 and 6.33 pmol/mg protein, respectively, compared to 19.82 pmol/mg protein for a clone optimally expressing human α1. The α2-clone, B, was used for all subsequent work. Figure 1C shows similar expression levels of the human α2, human α1 and pig α1 grown in 3 L culture spinner bottles. The membranes isolated from these clones also cross-reacted with an anti-β antibody (data not shown), showing the presence of the two bands described previously (see also Figure 3), and indicating parallel expression of the human α1 or α2 subunits and the porcine His_{10}-β1 subunits. Comparison of the Na,K-ATPase activity at 37 °C and ouabain binding of

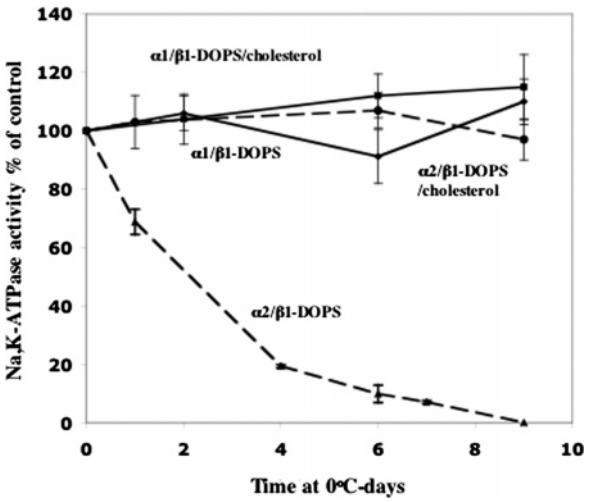

FIGURE 5: Stabilization of α2β1 at 0 °C by cholesterol in the presence of DOPS. α1β1 and α2β1 complexes were prepared in standard conditions with 0.1 mg/mL $C_{12}E_8$, and 0.05 mg/mL DOPS, without or with 0.01 mg/mL cholesterol. The protein was stored on ice, Na,K-ATPase activity (rate ± SE) at 37 °C was determined at the indicated times up to 9 days, and the percent of control ± SE was calculated.

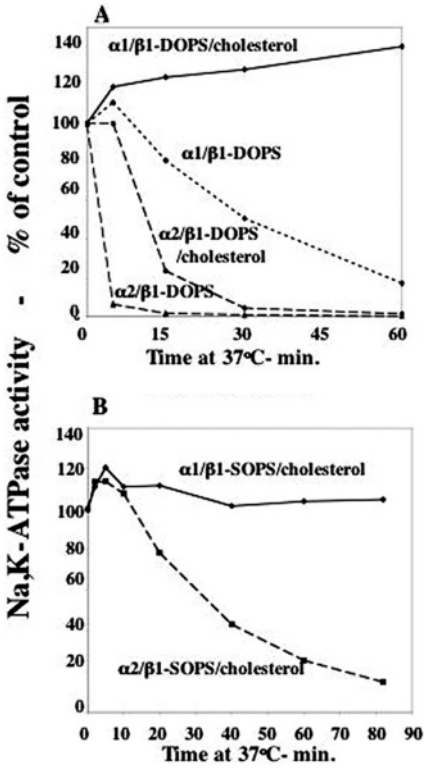

FIGURE 6: Stabilization of α1β1 and α2β1 at 37 °C by cholesterol in the presence of DOPS or SOPS. α1β1 and α2β1 complexes were prepared in standard conditions with 0.1 mg/mL $C_{12}E_8$, and (A) 0.05 mg/mL DOPS, without or with 0.01 mg/mL cholesterol or (B) 0.05 mg/mL SOPS with 0.01 mg/mL cholesterol. The protein was incubated at 37 °C for the indicated times, Na,K-ATPase activity was measured at 37 °C for each time point in duplicate, and the percent of control was calculated.

membranes expressing α1 and α2 allowed calculation of turnover numbers. The range for α1β1 was 4220−7200 min^{-1}, and in two experiments the values for α2β1 were 2449 and 3482 min^{-1} (average 2965 min^{-1}).

The thermal stability of the mature α1 and α2 proteins was tested by incubating membranes isolated from the α1-

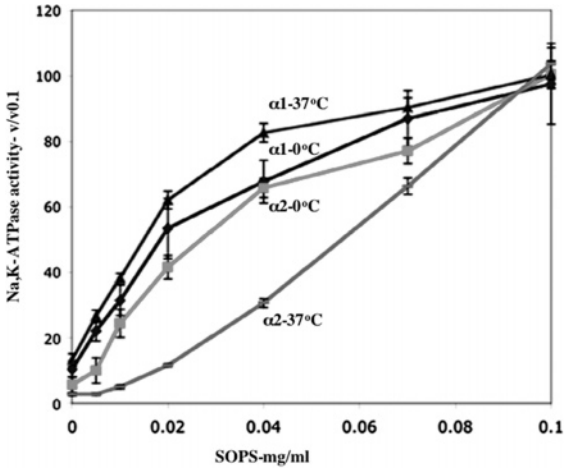

FIGURE 7: A difference in interaction of SOPS with α1β1 and α2β1 at 37 °C. Seven parallel preparations of α1β1 and α2β1 wre made in standard elution buffers containing 0.1 mg/mL $C_{12}E_8$, 0.01 mg/mL cholesterol, and 0, 0.05, 0.01, 0.02, 0.04, 0.07 and 0.1 mg/mL SOPS, respectively. Na,K-ATPase activity was measured immediately after elution from the beads or after a 7 min preincubation at 37 °C. Na,K-ATPase activities ± SE were estimated from slopes of the regression lines for P_i release (colorimetric assay). The Na,K-ATPase activity at each SOPS concentration was calculated relative to that 0.1 mg/mL SOPS and is presented as a percentage ± SE.

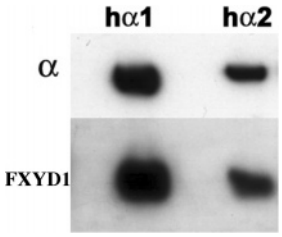

FIGURE 8: In vitro reconstitution of α1β1/FXYD1 and α2β1/FXYD1 complexes. Reconstitution of α1β1 and α2β1 with FXYD1 was done as described in the Experimental Procedures. The figure depicts immunoblots of eluted complexes probed with anti-KETTY (α) or anti-FXYD1 antibodies.

and α2-clones for up to 6 h at 37 °C, and then measuring oubain binding. As seen in Figure 2A, the ouabain binding capacity of both of the α1- and α2-clones was quite stable over 6 h at 37 °C. The temperature of the preincubation medium was then increased further to 45 °C. At this temperature the ouabain binding capacity of α2 fell progressively over 24 h, while that of α1 was unaffected (Figure 2B). This result indicates that α2 is intrinsically less thermally stable than α1, although both membrane-bound proteins are remarkably stable by comparison with the temperature-sensitivity of expression in the whole cells, or the purified detergent-soluble proteins, described below.

Membranes, prepared from cells expressing α2 in Figure 1C, were used to purify the human α2 porcine β1 complex, using the improved one-step procedure described recently for purification of the all porcine α1β1 and human α1 porcine β1 complexes (*43*) (see Figure 3). DDM is used for solubilization of membranes, but either $C_{12}E_8$ or DDM with an added phosphatidyl serine can be used for elution of the protein from the BD-Talon beads. As seen in Figure 3 (lane 4), the human α2 subunit was eluted together with the porcine β1 subunits, expressed as two bands, similar to the all porcine α1β1 and human α1 porcine β1 complexes (*42*, *43*). The purity and protein concentrations of the human α2

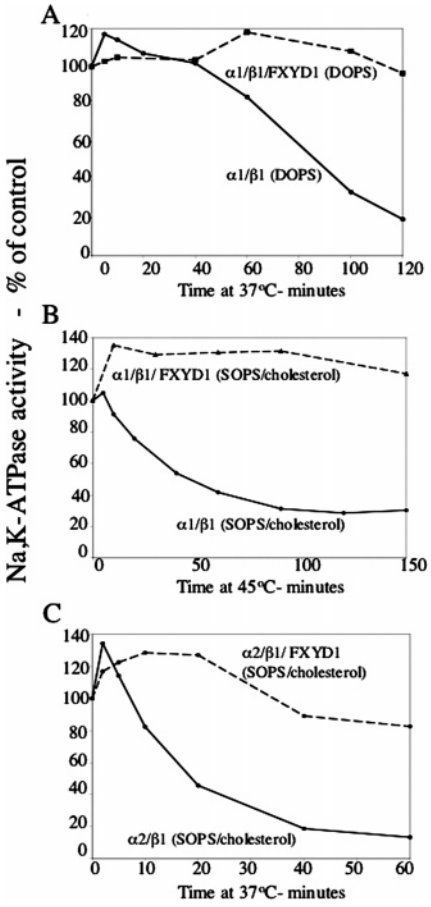

FIGURE 9: Stabilization of α1β1 and α2β1 by FXYD1. α1β1 complex was prepared with 0.1 mg/mL $C_{12}E_8$, and (A) 0.05 mg/mL DOPS or (B, C) 0.05 mg/mL SOPS with 0.01 mg/mL cholesterol. α2β1 complex was prepared with 0.05 mg/mL SOPS with 0.01 mg/mL cholesterol. Portions of the α1β1 and α2β1 preparations were reconstituted with FXYD1. The protein complexes were incubated for the indicated times at 37 °C (A, C) or 45 °C (B). Na,K-ATPase activity was then measured at 37 °C for each time point in duplicate, and the percent of control was calculated.

porcine β1 complex were comparable to those obtained for all porcine α1β1 and human α1 porcine β1. The ≈90 kDa band seen below the α subunit in some preparations is a minor contaminant and can be removed by raising the EDTA concentration in the binding buffer.

Functional Properties of Purified α1β1 and α2β1 Complexes. A phosphatidyl serine must be added to the purified recombinant Na,K-ATPase (porcine α1β1 or human α1 porcine β1) in order to stabilize the protein (*42*), and recent work shows that the asymmetric SOPS is optimal (*43*). As discussed extensively below, α2β1 is much less stable than α1β1 but, when prepared with SOPS/cholesterol, the purified α2β1 is stabilized.

The availability of stable α1β1 and α2β1 complexes, prepared with SOPS/cholesterol, has permitted comparison of the functional properties, and inhibition by ouabain, presented in Table 1. The Na,K-ATPase activity of α1β1 and α2β1 complexes prepared with SOPS/cholesterol was measured at 37 °C in conditions of maximal activity, and at varying ATP, Na and K concentrations. Two significant differences were found between the isoforms. First, the V_{max} of α2β1 (range 6−10 μmol/mg/min) was nearly always lower than that of α1β1 (range 8−16 μmol/mg/min). Second,

Table 1: Kinetic Parameters of Na,K-ATPase Activity of Purified α1β1 and α2β1 Complexes[a]

	V_{max} μmol/min/mg range	K_mATP ± SE (μM) ($n = 3$)	$K_{0.5}Na^+$, n_H ± SE (mM) ($n = 9$)	$K_{0.5}K^+$, n_H ± SE (mM) ($n = 4$)	$K_{0.5}$ ouabain ± SE (nM) ($n = 3$)
α1β1	8−16	222.6 ± 30.1	16.8 ± 0.89 n_H 1.6 ± 0.11	1.25 ± 0.03 n_H 1.91 ± 0.08	24.6 ± 6
α2β1	6−10	191.1 ± 33.8	15.7 ± 0.7 n_H 1.51 ± 0.8	2.30 ± 0.11 n_H 1.95 ± 0.14	102 ± 14

[a] The V_{max} values represent the range of specific activities observed in many experiments over a 2 year period. The values for $K_{0.5}$ Na^+, $K_{0.5}$ K^+ and K_mATP were obtained by fitting the average values of v/V_{max} to the functions $[X]^{nH}/([X]^{nH} + K_{0.5}{}^{nH})$ (X = Na, or K) or $[ATP]/([ATP] + K_m)$ respectively. For inhibition by ouabain, $K_{0.5}$ values were obtained by fitting average values of v/v_0 to the function $K_{0.5}/([ouabain] + K_{0.5})$.

the $K_{0.5}$ of K for α2β1 (2.30 ± 0.11 mM) was about double that for α1β1 (1.25 ± 0.03 mM). The kinetic parameters for activation by ATP and Na ions were not different between the α1β1 and α2β1 complexes. Inhibition by ouabain was assessed by adding the inhibitor directly to the Na,K-ATPase reaction medium at 37 °C (Table 1). Ouabain inhibited both isoforms at low concentrations, with slight selectivity toward α1β1 ($K_{0.5}$ 24.6 ± 6 nM) compared to α2β1 ($K_{0.5}$ 102 ± 14 nM). An important feature of such experiments is that the reaction time must be sufficiently long for the inhibitor to fully equilibrate with the protein. Control experiments showed that, at any concentration of ouabain, the degree of inhibition of α1β1 was constant only after 40 min, whereas inhibition of α2β1 was constant even after 10 min. Indeed, $K_{0.5}$ values for inhibition by ouabain over 10 and 60 min reaction times, respectively, were 460 ± 70 ($n = 10$) versus 24.6 ± 6 nM ($n = 3$) for α1β1, and 98 ± 9 ($n = 5$) versus 102 ± 14 nM ($n = 3$) for α2β1. Thus, 60 min reaction times were used for all inhibition experiments. The difference between human α1 and α2 reflects a more rapid equilibration of ouabain with α2, as documented previously in direct ouabain binding assays (*38*).

Instability of Purified of α2β1. Stabilization by SOPS and Cholesterol. Although the α1β1 and α2β1 complexes eluted in the conditions of Figure 3 (0.1 mg/mL $C_{12}E_8$/and 0.05 mg/mL DOPS) were both active immediately after elution, it soon became clear that the α2β1 complex is easily inactivated. In an initial indication, we tested whether it is necessary to add DOPS dissolved in $C_{12}E_8$ to the Na,K-ATPase reaction medium (Figure 4). The protein, detergent and lipid are all diluted about 100-fold into the reaction medium and, in the absence of added $C_{12}E_8$/DOPS, ATP hydrolysis of both α1β1 and α2β1 was linear over time. This shows that both α1β1 and α2β1 are stable at 37 °C in these conditions. Addition of $C_{12}E_8$/DOPS had little or no effect on the Na,K-ATPase activity of α1β1. Since both the detergent and lipid are diluted about 100-fold, to a concentration below the cmc of $C_{12}E_8$, maintenance of Na,K-ATPase activity has the important implication that DOPS is still bound to the protein, as discussed previously (*42*, *43*). By contrast to the lack of effect on α1β1, addition of $C_{12}E_8$/DOPS to the reaction medium completely inactivated the α2β1 complex. The experiment in Figure 4 clearly suggests that the α2β1 complex is sensitive to the detergent. Rapid inactivation by the $C_{12}E_8$ was also demonstrated directly by comparing the thermal stability when eluted in media containing a fixed concentration of DOPS and varying concentrations of $C_{12}E_8$ (not shown).

Figures 5 and 6 compare stability of α1β1 and α2β1 in different conditions and means to stabilize the α2β1 complex. Figure 5 shows that upon storage of the α2β1 complex at 0 °C in the elution conditions used previously for purification of α1β1 (0.1 mg/mL $C_{12}E_8$ and 0.05 mg/mL DOPS), the Na,K-ATPase activity is lost with a half-time of 1−2 days. Due to this instability, the purified α2β1 complex cannot be stored and studied systematically in these conditions. However, the crucial observation is that addition of a low concentration of cholesterol to the DOPS preserved activity of α2β1 stored at 0 °C for up to 9 days. Other experiments showed that 0.01 mg/mL cholesterol sufficed for a maximal stabilizing effect. Note that, by contrast, the Na,K-ATPase activity of the α1β1 prepared with DOPS is stable at 0 °C and thus no additional stabilization by cholesterol is detectable (confirming an observation in (*43*)). Although cholesterol is not required to preserve activity of human α1 porcine β1 or of all porcine α1β1 at 0 °C, addition of cholesterol together with DOPS strongly stabilizes the porcine α1β1 complex at 37 °C (*43*). Figure 6A confirms the strong stabilizing effect of cholesterol at 37 °C on the α1β1. Figure 6A also shows that α2β1 complex prepared with DOPS alone was very rapidly inactivated at 37 °C ($t_{1/2}$ 1−2 min) and, again, cholesterol protected significantly ($t_{1/2}$ ca. 10 min). Other lipid combinations such as DOPC and DOPC/cholesterol have also been looked at, but are much less effective stabilizers than DOPS/cholesterol (not shown). The asymmetric SOPS stabilizes both all porcine α1β1 and human α1 porcine β1 significantly better than the symmetric DOPS (*43*), and a similar difference has also been observed for the α2β1 complex (not shown). The experiment in Figure 6B shows that the combination of SOPS/cholesterol stabilizes α2β1 significantly better at 37 °C than does DOPS/cholesterol (compare the $t_{1/2}$ of 30−40 min with $t_{1/2}$ ca. 10 min, seen in Figure 6A). It is also seen that, by comparison with α2β1, the α1β1 complex prepared with SOPS/cholesterol is even more stable, the activity being preserved intact over 80 min at 37 °C. In other experiments it was shown that α2β1, prepared with SOPS/cholesterol, loses activity at 0 °C with a half-time of about 20 days, and also that the plant sterol ergosterol is much less effective than cholesterol (not shown). Thus, SOPS/cholesterol is the optimal lipid combination for storage and stabilization of α2β1. When both isoforms are prepared with SOPS/cholesterol, systematic study of α2β1 and comparison with α1β1 becomes possible.

Figure 7 provides a direct demonstration that the different stabilities of α1β1 and α2β1 at 37 °C are related to a difference in interaction with SOPS. Each isoform was prepared with 0.1 mg/mL $C_{12}E_8$, 0.01 mg/mL cholesterol and

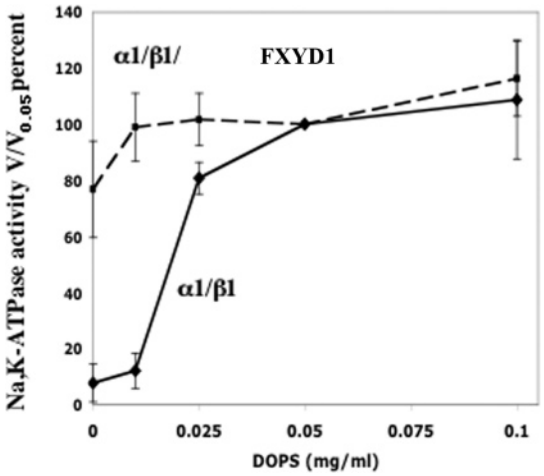

FIGURE 10: Dependence of Na,K-ATPase activity of $\alpha1\beta1$ and $\alpha1\beta1$/FXYD1 on added DOPS. The $\alpha1\beta1$ complex was or was not reconstituted with FXYD1. The BD-Talon beads were then washed and the protein complexes were eluted in buffers containing 0.1 mg/mL $C_{12}E_8$ and 0−0.1 mg/mL of DOPS. The Na,K-ATPase activity was measured for each preparation, the value $v/v_{0.05}$ calculated, and then average values of $v/v_{0.05} \pm$ SE calculated for replicate experiments ($n = 4-7$).

varying concentrations of SOPS between 0 and 0.1 mg/mL. The Na,K-ATPase activity was then measured immediately after elution of the complexes at 0 °C, or after a prior incubation at 37 °C. The result is quite clear-cut in that the dependence of activity on SOPS concentration is correlated with stability. At 0 °C, both isoforms are stable in the presence of cholesterol (Figure 5) and the dependencies on SOPS concentration are similar, with a slightly higher "apparent affinity" for $\alpha1\beta1$. However, after a 37 °C incubation, the curve for $\alpha2\beta1$ is strongly shifted to the right, while that for $\alpha1\beta1$ is shifted somewhat to the left. The result is a large difference of "apparent affinity" between $\alpha1\beta1$ and $\alpha2\beta1$, showing that much higher concentrations of SOPS are required to protect $\alpha2\beta1$ against thermal inactivation at 37 °C, compared to $\alpha1\beta1$. This finding is quite consistent with the large absolute difference in stability of $\alpha1\beta1$ and $\alpha2\beta1$ presented in Figure 6B.

Stabilization of Purified $\alpha1\beta1$ and $\alpha2\beta1$ by FXYD1. Incubation of the porcine $\alpha1\beta1$ complex with human FXYD1 (phospholemman) allows spontaneous reconstitution of the $\alpha1\beta1$/FXYD1 complex, which can then be used for functional characterization (*46*). The experiments in Figures 8−10 present evidence for reconstitution and strong protection by FXYD1 against thermal inactivation of both $\alpha1\beta1$ and $\alpha2\beta1$ complexes, and some insight into the mechanism.

The immunoblot in Figure 8 shows that human FXYD1 associates spontaneously *in vitro* with either $\alpha1\beta1$ or $\alpha2\beta1$, bound to the BD-Talon beads, thus reconstituting the $\alpha1\beta1$/FXYD1 and $\alpha2\beta1$/FXYD1 complexes. Increasing the amount of FXYD1 added to the beads did not increase the amount of FXYD1 eluted with the $\alpha1\beta1$ and $\alpha2\beta1$ complexes, suggesting that the FXYD1 associates stoichometrically with both α1 and α2 isoforms. A large fraction of FXYD1 added to the beads remained unbound, consistent with the assumption that the FXYD1 was present in molar excess (not shown). In Figure 8, it can also be seen that there is also an approximate proportionality between the amount of α1 or α2 subunit and amounts of associated FXYD1, detected with the specific antibodies, suggesting again that FXYD1 binds stoichiometrically to both $\alpha1\beta1$ and $\alpha2\beta1$ complexes.

Figure 9 compared the thermal stability of $\alpha1\beta1$ with $\alpha1\beta1$/FXYD1 and $\alpha2\beta1$ with $\alpha2\beta1$/FXYD1 complexes. As seen in Figure 9A the $\alpha1\beta1$ complex prepared in 0.1 mg/mL $C_{12}E_8$/ 0.05 mg/mL DOPS was progressively inactivated at 37 °C, while that of $\alpha1\beta1$/FXYD1 was fully preserved during the 2 h incubation. Since SOPS/cholesterol is itself strongly stabilizing, by comparison with the DOPS, it was of interest whether the FXYD1 would further stabilize $\alpha1\beta1$/FXYD1 prepared with SOPS/cholesterol. The $\alpha1\beta1$ and $\alpha1\beta1$/FXYD1 complexes were prepared in the presence of 0.04 mg/mL SOPS and 0.01 mg/mL cholesterol 0.08 mg/mL $C_{12}E_8$, and were both found to be fully active after 160 min of preincubation at 37 °C (not shown). Thus, in order to test a possible additional stabilizing effect of FXYD1 the temperature was raised 45 °C. As seen in Figure 9B, at 45 °C about 75% of the Na,K-ATPase activity of $\alpha1\beta1$ was lost after 100 min while activity of $\alpha1\beta1$/FXYD1 was fully preserved even after 150 min preincubation at 45 °C. Finally, as seen in Figure 9C, FXYD1 also strongly stabilizes $\alpha2\beta1$. The $\alpha2\beta1$ complex prepared in SOPS/cholesterol lost about 80% of its Na,K-ATPase activity during a 60 min incubation at 37 °C, while activity of the reconstituted $\alpha2\beta1$/FXYD1 was largely preserved. To summarize, stabilization by FXYD1 does not seem to be α1- or α2-specific, since both $\alpha1\beta1$ and $\alpha2\beta1$ complexes are strongly protected against thermal inactivation.

An important clue to the mechanism of the stabilizing effect of FXYD1 was obtained in the experiment depicted in Figure 10. The control $\alpha1\beta1$ complex or reconstituted $\alpha1\beta1$/FXYD1 complexes were eluted from BD-Talon beads in the presence of different concentrations of DOPS (0, 0.01, 0.025, 0.05 and 0.1 mg/mL DOPS dissolved in 0.1 mg/mL $C_{12}E_8$). The results are presented as the ratio of rates at each lipid concentration compared to that at 0.05 mg/mL, as a percentage, and represent averages ± SEM of 4−7 experiments. Consistent with the previous observation, Figure 10 shows that $\alpha1\beta1$ is inactive in the absence of an added PS, and the activity of the complex increases to a plateau as the DOPS concentration was increased up to 0.05 mg/mL and above (*42*, *43*). Strikingly, however, the $\alpha1\beta1$/FXYD1 complex was largely active even without added DOPS, and added DOPS increased the rate by 20% at the most (see Discussion for a likely explanation).

Oligomeric Structure of Purified $\alpha1\beta1$ and $\alpha2\beta1$ Complexes. A possible explanation of the differences in stability of the α1 and α2 isoforms, and stabilizing effects of FXYD1, is that the oligomeric structures differ. In principle, higher order oligomers could confer stability. In order to test this possibility, the detergent-soluble complexes have been analyzed by size-exclusion chromatography (Figure 11). The HPLC protein profiles of the eluted $\alpha1\beta1$ and $\alpha2\beta1$ complexes are essentially identical.[2] The major peak of both $\alpha1\beta1$ and $\alpha2\beta1$ complexes in these conditions is the $\alpha\beta$ protomer, together with a significant shoulder corresponding to a mixture of higher order oligomers. The immunoblot in

[2] In the current experiments, a PS was not added to the HPLC running buffers because our previous work showed that the porcine $\alpha1\beta1$ eluted at the same position when a low concentration of DOPS was added to the elution buffer containing DDM (*42*).

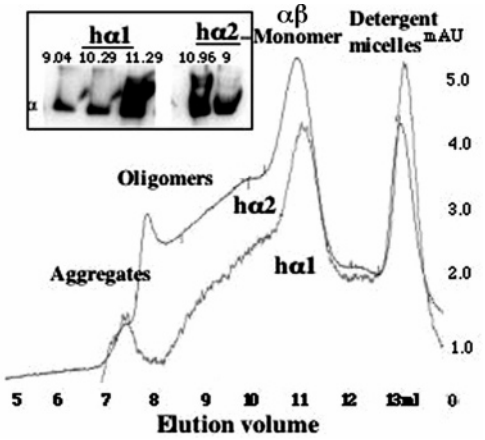

FIGURE 11: Size-exclusion HPLC of α1β1 and α2β1 complexes. 50−100 μg of α1β1 and α2β1 complexes prepared with 0.1 mg/mL $C_{12}E_8$, 0.05 mg/mL SOPS, 0.01 mg/mL cholesterol were injected onto the HPLC column. Fractions were collected at the elution volumes corresponding to the $\alpha\beta$ peak or preceding shoulder, and aliquots were loaded on gels for Western blot analysis, using anti KETTY antibodies (inset).

the inset shows that the α subunit is found in both the major peak and the shoulder. The assignment of the major peak as the $\alpha\beta$ protomer was made previously, based on apparent mass measurement estimated from standard proteins and detergent binding data (*42*, *43*). Other experiments showed also that FXYD1 does not affect the HPLC profiles (not shown). Thus, the clear conclusion is that neither the difference in stability of α1 and α2 nor stabilization of α1 and α2 by FXYD1 can be attributed to different oligomeric structures.

DISCUSSION

Expression of the Human α2 *Isoform.* Optimal expression of the human α2 isoform required reduction of the temperature of the methanol induction phase to 20 °C. By contrast, α1 is not as sensitive to temperature and is expressed optimally at 25 °C (*41*). The necessity of growth of *P. pastoris* expressing α2 at 20 °C fits well with the observations that α2 expressed in *S. cerevisiae* is quite unstable, compared to α1 and α3 isoforms, and is expressed at much lower levels (α1, 7.4; α2, 0.8; α3, 5.6 pmol/mg protein) (*39*). Optimal expression of α2 at 20 °C in the *P. pastoris* (15.3 pmol ouabain bound/mg protein) was almost 20-fold higher than that described previously for *S. cerevisiae* (0.8 ouabain bound pmol/mg) (*39*).

A likely explanation of temperature sensitivity of expression of α2 is that, during synthesis, trafficking, or membrane-insertion above 20 °C, α2 is destabilized and unfolded, relative to α1, and is susceptible to complete proteolytic degradation. Destabilization of α2 could result from suboptimal interactions with phospholipids in the endoplasmic reticulum or trafficking vesicles. In principle, temperature sensitivity of expression might also result from instability and degradation of the mature membrane-inserted protein, although the experiment of Figure 2 shows that the membrane-bound α1 and α2 isoforms are both remarkably stable (Figure 2A). Nevertheless, at an elevated temperature of 45 °C an intrinsic difference in stability of α2 versus α1 was revealed (Figure 2B). Since specific interactions with phosphatidyl serine/cholesterol are crucial for stabilization of both native renal and recombinant Na,K-ATPase (*42*, *53*), the fact that the environment of intact yeast membranes strongly stabilizes both α1 and α2 (Figure 2) suggests that the native phospholipids play an important role. *P. pastoris* membranes contain high concentrations of PS (mainly DOPS) (G. Daum, University of Vienna, personal communication) and, in the absence of a delipidating agent such as a detergent, both isoforms should associate with the native PS in the intact membrane and be protected against thermal inactivation. Nevertheless, the relative instability of α2, compared to α1 (seen at 45 °C in Figure 2), may still reflect a difference in interaction with the native PS. In addition, since yeast membranes contain ergosterol rather than cholesterol, which is the major sterol of mammalian membranes, and ergosterol is a poor stabilizer of α2 (unpublished), the lack of cholesterol may also contribute to the difference in stability of α1 and α2.

Purification of the α1β1 *and* α2β1 *Complexes. Functional Properties and Oligomeric Structure.* The relatively high expression level of the α2 isoform has allowed purification of the human α2 porcine β1 to about 80%, approximately the same degree as we have reported recently for either porcine α1β1 or human α1 porcine β1 (*43*). Stabilization of the purified α2β1 complexes with SOPS/cholesterol has provided a preparation, of which the kinetic properties and inhibitor sensitivity can be compared with those of α1β1. The differences in kinetic properties of the α2β1 and α1β1 include a somewhat lower V_{max} and higher $K_{0.5}$K for α2, but no significant difference for $K_{0.5}$Na and K_mATP (Table 1). The lower V_{max} for α2 was not unexpected because the turnover number of α2β1 in the membrane-bound form (2965 min^{-1}, average of two experiments) was also found to be lower than for α1β1 (range 4220−7200 min^{-1}).[3] In *Xenopus oocytes*, a lower turnover rate and higher $K_{0.5}$K were also observed for α2 compared to α1 and a slightly higher $K_{0.5}$Na (*38*). For the human isoforms expressed in *S. cerevisiae* ligand binding affinities were compared from effects on ouabain binding (*39*). Although the values are not strictly comparable to values obtained from ligand-dependence of Na,K-ATPase activity, these studies also showed a significantly higher $K_{0.5}$K, a somewhat higher $K_{0.5}$Na and no difference in K_mATP, for α2 compared to α1.

Inhibition of Na,K-ATPase activity of α1β1 and α2β1 complexes by ouabain confirms that both isoforms have a high affinity for the inhibitor, and show a slight selectivity of ouabain for α1 (Table 1). A faster rate of equilibration with α2 can also be inferred from the fact that inhibition becomes constant at shorter reaction times at 37 °C, compared to inhibition of α1, as described in the Results. These features are consistent with ouabain binding to the isoforms expressed in *Xenopus oocytes* (*38*) and *S. cerevisiae* (*39*). In *Xenopus oocytes*, in particular, much faster rates of ouabain binding and dissociation were observed for α2 compared to α1 (*38*). In summary, the similarities and

[3] It could be asked whether properties of human α porcine β complexes are identical to all human or all porcine $\alpha\beta$ complexes. We have shown that the Na,K-ATPase activities of purified human α1 porcine β1 and all porcine α1β1 complexes are not significantly different (*43*). Also, we have recently expressed all human α2β1 in *P. pastoris*. A Na,K-ATPase turnover number in membranes of 2409 min^{-1} has been measured, i.e., a value quite similar to that observed with human α2 porcine β1 (E. Dinitz and S.J.D.K., unpublished).

differences between kinetic properties, and inhibition by ouabain, detected for the purified detergent-soluble α1β1 and α2β1 complexes are quite similar to those observed for the membrane-bound α1 and α2 isoforms expressed in *Xenopus oocytes* and *S. cerevisiae*.

The oligomeric structure of the α1β1 and α2β1 complexes in $C_{12}E_8$ is heterogeneous, but consists largely of the αβ protomer, and there is no significant difference between the isoforms. Our recent work shows that the proportion of higher $(\alpha1\beta1)_n$ oligomers can be increased by concentrating the protein, but the oligomers are unspecific as they are easily dissociated to αβ protomers by low concentrations of DDM, or by dilution. The Na,K-ATPase activity is the same in $C_{12}E_8$ and DDM, and the oligomeric state does not confer stability, i.e., oligomers play no specific role in function or stability (*43*). In previous studies it was observed that thermal inactivation of renal Na,K-ATPase activity in the non-ionic detergent is associated with aggregation of the protein (*54*). The work with the detergent-soluble renal and recombinant Na,K-ATPase shows that instability is caused primarily by detergent-mediated delipidation of the protein, and is probably associated with disruption of α/β subunit interactions (*42*, *43*). As shown by HPLC in the absence of added PS, inactivation occurs without a change in the aggregation state (*42*, *53*). Thus, inactivation may be followed by protein aggregation over time. In summary, the current experiments show clearly that (a) the minimal functional unit of the α2, like the α1, isoform is the αβ protomer, (b) oligomers play no specific role in function or stability of α2 in the detergent-soluble state, although a role in the membrane-bound state is not excluded, as discussed previously in relation to α1 (*42*, *43*) and (c) the large difference in stability between α1 and α2, without or with FXYD1, cannot be explained by a differences in the oligomeric structure.

The Origin of Instability of α2. Stabilization by Lipids and FXYD1. By far the most striking difference in properties of the α1 and α2 isoforms yet observed is the thermal instability of the α2β1 complex. This difference in stability of α1 and α2 is detected in whole cells (Figure 1), in intact membranes (Figure 2B), and in the detergent-soluble purified complexes (Figures 4−7), even though the absolute stability varies greatly in the different conditions. Based on our observations on stabilization of the purified recombinant α1β1 complex, phosphatidyl serine and cholesterol were proposed to interact specifically with the protein and each other, near the α and β subunit interface, and protect it against detergent-mediated delipidation and thermal inactivation (*42*, *43*).

The results of this study show that instability of α2β1 is attributable to suboptimal phospholipid−protein interactions. Indirect evidence for this conclusion is that the purified α2β1 complex is much more sensitive to detergent than α1β1 (Figure 4), and it is specifically stabilized by cholesterol at 0 °C, whereas α1β1 is stable at 0 °C even without cholesterol (Figure 5). As we have discussed recently (*43*), the detergent inactivates by displacing bound PS, and the cholesterol stabilizes the interaction of the bound PS with the protein. Direct evidence for a difference in SOPS−protein interaction was obtained in the experiment (Figure 7) showing that much higher concentrations of SOPS are required to protect α2 against thermal inactivation compared to α1.

A striking insight into the possible origins of α2-instability can be obtained from sequence comparisons of α2 with α1 and α3, and a finding that α2 is uniquely unstable, compared to both α1 and α3, which show similar stabilities (*39*). There are twenty-eight residues in α2, which are not found in either α1 or α3. When mapped onto a model of α1 (Figure 12) these residues are seen to be located primarily to two regions.[4] First, most of the residues are in the N domain, and are unlikely to be involved in lipid−protein interactions. Second, there are only three residues unique to α2 in trans-membrane segments, namely in M8 (α1-V919; α2 A920), M9 (α1-F954; α2-L955) and M10 (α1-P980; α2-V981), and one residue in the extracellular loop L1−2 near M1 (α1-T116; α2 =M118). M118 in L1−2 is known to play an important role in ouabain binding and the high rates of ouabain binding and dissociation of α2 (*38*, *55*), and so it is probably not involved in lipid−protein interactions. By contrast, the residues in M8, M9 and M10 mapped in α2 are of great interest. Based on cryoelectron microscopy of renal Na,K-ATPase, the trans-membrane segment of the beta subunit (Mβ) has been located within the groove between M8, M9 and M10 (*56*, *57*), and cross-linking evidence also positions it near M8, (*58*, *59*), although a position between M7 and M10 is also possible (*60*). Thus, the fact that the three α2-specific residues are located in M8, M9 and M10

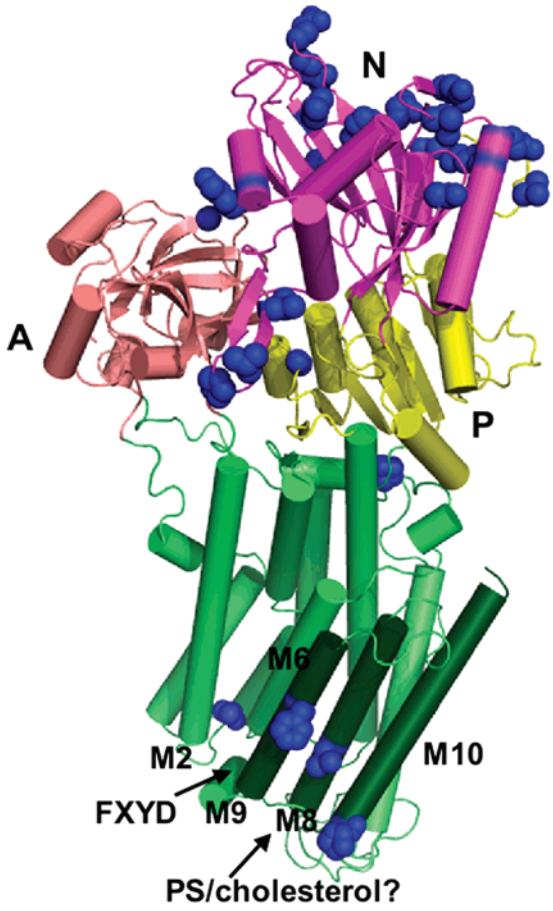

FIGURE 12: Homology model showing residues unique to α2. The homology model of the porcine α1 subunit is based on the SERCA molecular structure (1VFP.pdb) see (*62*). Residues (in blue) shown in M8, M9 and M10 are V919, F954, and P980 respectively. Other residues shown (in blue) include T116 in L1/2 and twenty residues mostly in the N domain. The model was drawn with PYMOL.

[4] Four of the 28 unique α2 residues cannot be mapped because they are located in the cytoplasmic N-terminus, which is not present in the homology model.

supports the proposal that the PS (head group and fatty acyl chains) interact near the α*β* subunit interface boundary, mediating the subunit interactions and stabilizing the protein (*43*). In the case of α2*β*1, the interactions of the PS could be less optimal leading to suboptimal α*β* subunit interactions and destabilization of the protein. Since the PS head group normally faces the cytoplasmic leaflet of the bilayer (*61*), these residues might interact primarily with the fatty acyl chains of the PS. In any event, it will be necessary to test the hypothesis experimentally. One could expect that mutation of these residues in α2 to those in α1 would stabilize the protein.

Another significant prediction of the model in Figure 12 is that other trans-membrane segments are not involved in the different stability of α1 and α2. In this context, it is of interest that the trans-membrane segments of FXYD proteins have been located in the groove between M2, M6 and M9, adjacent to but distinct from the proposed location of bound PS (*46*, *56*, *57*, *62*, *63*). Based on the model, one would not predict differential stabilization of α1 and α2 by FXYD1, consistent with the findings in Figure 9. Proximity of bound FXYD1 and phospholipids might also explain simply the result in Figure 10. Preservation of Na,K-ATPase activity of the α1*β*1/FXYD1 complex without addition of exogenous PS implies either that FXYD1 replaces the added PS or that the FXYD1 stabilizes the interaction of the endogenous *P. pastoris* PS with the protein. However, the first assumption does not fit with evidence for simultaneous binding of FXYD1 and phosphatidyl serine, such as that in Figure 9, showing that the presence of either less (DOPS) or more (SOPS/cholesterol) strongly stabilizing lipids does not preclude additional stabilization by FXYD1. In addition, detergent-soluble renal Na,K-ATPase, which includes bound FXYD2, is known to be stabilized by added PS (*53*). Also, very recent work shows that interactions of PLMS with the shark Na,K-ATPase are affected by anionic lipids (*64*). Thus, it is more likely that FXYD1 stabilizes by interacting with bound PS. Since the trans-membrane segment of FXYD1 lies close to M9, positively charged residues at the membrane−water interface (e.g. RRCRCK) or in a short cytoplasmic helix FRSSIRRLS, see (*65*), could interact with the negative charge of the phosphate of PS.

Physiological Implications? Although the objective of this study has been to purify and stabilize the α2 isoform, the results raise interesting questions related to the physiological roles of α2 and effects of FXYD1. For example, the α2-specific residues mapped to the cytoplasmic domains in Figure 12 (N domain and N-terminus) are located in ideal positions for interactions with the cytoskeletal proteins, such as ankyrin. For brain astrocytes and arterial myocytes, there is histochemical evidence for colocalization of cytoskeleton with the α2 isoform and other Ca transporting proteins in microdomains overlying the ER (*25*−*29*, *35*) and more direct evidence in brain tissues for interactions between α2 (but not α1), with NCX1, with ER proteins such as SERCA2 and the IP3 receptor, and ankyrin2 (*66*). It is proposed that the macromolecular complexes are particularly efficient in Ca signaling. In cardiac myocytes, both α1 and α2 isoforms have been located within T-tubules, in a macromolecular complex with NCX1, IP3 receptor, mediated by ankyrin-B, and an important role in cardiac Ca signaling of both isoforms in the macromolecular complex has been proposed (*67*). Other recent work has demonstrated differential localization of α1 and α2 isoforms in cardiac T-tubules and surface sarcolemma membranes, respectively, and assigns a more prominent role for α2 in Ca signaling (*31*, *32*). A second possibility of interest is that the α2-specific residues mapped to the trans-membrane segments M8, M9, and M10 are normally involved in interactions with the lipid environment. The stabilizing effect of cholesterol on α2, observed in this study could be indicative of a similar stabilizing role *in vivo*. In addition, there is now quite substantial evidence for localization of the Na,K-ATPase in isolated caveolae from cardiac mycocytes and epithelial cells, and association with caveolin, respectively (*68*−*70*). Caveolae are specific forms of cholesterol- and sphingolipid-rich lipid rafts (*71*), and it has been reported that depletion of cholesterol depletes the caveolar Na,K-ATPase (*69*). Thus, the effect of cholesterol on α2 might also mimic a specific interaction of cholesterol, which affects association of α2 with lipid microdomains and its stability.

Detailed functional effects of FXYD1 on Na,K-ATPase activity of the purified isoforms, including effects of phosphorylation at Ser68, will be described in a separate paper. The observations that FXYD1 spontaneously associates with the α/*β* complexes (Figure 8) and strongly protects against thermal inactivation (Figure 9) provide evidence for specific interactions with both human α1 and α2 isoforms. Do they have physiological significance? The result is consistent with some previous findings but not with others. For example, when expressed in *Xenopus oocytes* FXYD1 affects kinetics of Na,K-pump currents of both α1 and α2 isoforms (*47*), but in guinea pig ventricular myocytes it appears that α2 does not interact functionally with FXYD1 (*30*). As judged by co-immunoprecipitation, FXYD1 interacts physically with both α1 and α2 in native bovine sarcolemma vesicles (*47*), albeit more weakly with α2, but interacts only with α1 in guinea pig myocyte membranes (*30*). Different cellular locations of α1, α2 and FXYD1 might account for lack of interactions of FXYD1 with α2 in myocytes, but the discrepancies could also reflect species differences. Observations in FXYD1 knock-out mice are of interest in the present context (*72*). The FXYD1-deficient heart showed hypertrophy, and decreased Na,K-ATPase activity of the cardiac sarcolemma, due partly to a lower turnover rate and partly to lowered expression of α1 and α2. α2 has been reported to be down-regulated in various forms of hypertrophy (*13*, *73*−*75*), and one possibility is that reduced expression is secondary to some hypertrophy program and not due to the absence of FXYD1. However, it is also possible that the absence of FXYD1 destabilizes both α1 and α2, but especially the intrinsically unstable α2, and thus makes it more susceptible to cellular degradation.

Another relevant finding is that the renal Na,K-ATPase from FXYD2-deficient mice is thermally labile, suggesting that FXYD2 stabilizes the protein (α1*β*1) (*76*). The evidence for protection against thermal inactivation of Na,K-ATPase by both FXYD1, in this work, and FXYD2 suggests that stabilization could be a common feature of interactions of FXYD proteins with the pump in physiological conditions, in addition to modulation of the transport kinetics.

Perspective. The purified, functional and stable α2*β*1 complexes are available in relatively large quantities (0.1−1 mg) and are suitable for detailed biochemical and biophysical

studies. The preparation might also be very useful in high throughput screens and development of new α2-specific inhibitors. Cardiac glycosides are dangerous drugs, and α2-selective inhibitors could have the beneficial positive inotropic but reduced toxic effects. With further stabilization of the α2β1 complex, structural work might also become feasible.

In a more general context, it has been pointed out that of the few recombinant integral membrane proteins, which have been crystallized, several have been expressed in yeast, including Ca-ATPase (*77*), and in each case extensive prior optimization of expression, purification and stabilization have been essential (*78*). The present work shows that it cannot be assumed that precisely the same conditions will be appropriate for purification of different isoforms of membrane proteins. Rather, optimization of expression, purification and stabilization of each similar but nonidentical protein species is an important task in itself.

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