Purification of Na\textsuperscript{+},K\textsuperscript{+}-ATPase Expressed in *Pichia pastoris*
Reveals an Essential Role of Phospholipid-Protein Interactions*

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Na\textsuperscript{+},K\textsuperscript{+}-ATPase (porcine a/\text{his}_{\text{a}},\beta/b\text{hi}) has been expressed in *Pichia Pastoris*, solubilized in n-dodecyl-\text{β}-maltoside and purified to 70–80% purity by nickel-nitrilotriacetate acid chromatography combined with size exclusion chromatography. The recombinant protein is inactive if the purification is done without added phospholipids. The neutral phospholipid, dioleoylphosphatidylcholine, preserves Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity of protein prepared in a Na\textsuperscript{+}-containing medium, but activity is lost in a K\textsuperscript{+}-containing medium. By contrast, the acid phospholipid, dioleoylphosphatidylserine, preserves activity in either Na\textsuperscript{+}- or K\textsuperscript{+}-containing media. In optimal conditions activity is preserved for about 2 weeks at 0 °C. Both recombinant Na\textsuperscript{+},K\textsuperscript{+}-ATPase and native pig kidney Na\textsuperscript{+},K\textsuperscript{+}-ATPase, dissolved in n-dodecyl-\text{β}-maltoside, appear to be mainly stable monomers (α/β) as judged by size exclusion chromatography and sedimentation velocity. Na\textsuperscript{+},K\textsuperscript{+}-ATPase activities at 37 °C of the size exclusion chromatography-purified recombinant and renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase are comparable but are lower than that of membrane-bound renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase. The β subunit is expressed in *Pichia Pastoris* as two lightly glycosylated polypeptides and is quantitatively deglycosylated by endoglycosidase-H at 0 °C, to a single polypeptide. Deglycosylation inactivates Na\textsuperscript{+},K\textsuperscript{+}-ATPase prepared with dioleoylphosphatidylcholine, whereas dioleoylphosphatidylserine protects after deglycosylation, and Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity is preserved. This work demonstrates an essential role of phospholipid interactions with Na\textsuperscript{+},K\textsuperscript{+}-ATPase, including a direct interaction of dioleoylphosphatidylserine, and possibly another interaction of either the neutral or acid phospholipid. Additional lipid effects are likely. A role for the β subunit in stabilizing conformations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (or H\textsuperscript{+},K\textsuperscript{+}-ATPase) with occluded K\textsuperscript{+} ions can also be inferred. Purified recombinant Na\textsuperscript{+},K\textsuperscript{+}-ATPase could become an important experimental tool for various purposes, including, hopefully, structural work.

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase is a member of the P-type ATPase family of active cation pumps. Na\textsuperscript{+},K\textsuperscript{+}-ATPase utilizes the free energy of hydrolysis of ATP to actively transport three intra-

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P. pastoris, with ovalbumin binding capacity of about 30 pmol/mg of protein, is a great advantage over the other expression systems. The availability of sufficient material has allowed exploration of optimal experimental procedures to obtain a purified, stable, and functional preparation. This report describes purification of the recombinant Na\(^+\),K\(^+\)-ATPase and provides evidence for specific phospholipid-protein interactions, the oligomeric state of the protein, and role of the β subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—P. pastoris protease-deficient strain SMB-165 (his4, prb1) was used for transformation. BMG and BMM media were prepared from YNB medium (Difco) as described in Ref. 20. DMM (Anagrade catalog no. D310) was obtained from Anatrace. Synthetic DOPC (catalog no. 850375) and DOPS (catalog no. 830035) were obtained from Avanti and stored as chloroform solutions. Recombinant Endo-H (400,000 units/mg, catalog no. P07025) was obtained from New England Biolabs. Ni\(^{2+}\)-NTA beads were from Qiagen.

**Construction of Expression Vectors**—pHIL-D2(a/his\(_{10}\), pHL-D2(his\(_{10}\)/his\(_{10}\)), and pHIL-D2(his\(_{10}\)/β)—DNAs encoding porcine α1 (accession X03938) in plasmid pGEM and porcine β1 (accession X04635) in plasmid PBR322 were cloned into the expression vector pHIL-D2/β (1533 bp) by standard procedures (see Ref. 20). Ten histidine residues have been added to the N-terminus of the α-subunit pHIL-D2(a/his\(_{10}\)) or the N-terminus of the β subunit pHIL-D2 (a/His\(_{10}\)/β or both) of DDM (a/His\(_{10}\)/β). The His tag coding sequence was ligated to the α and β subunit by means of ApaI and BglII restriction sites, respectively, engineered by site-directed mutagenesis. The nucleotide and amino acid sequences of the His tag at the N terminus of the β subunit (a/his\(_{10}\)), the only tagged construct leading to expression of protein, are as follows: atg gca gca ttc gca gaa gca ASh HHH HHH HHH PRR SRG.

**Yeast Transformation, Selection, and Growth**—10 μg of linear DNA obtained by digestion of plasmids pHIL-D2(a/his\(_{10}\)), pHIL-D2(his\(_{10}\)/β), or pHIL-D2(his\(_{10}\)/β/β) with NotI, were used to transform spheroplasts of P. pastoris SMB1165, and His\(_{+}\), Mut\(^{-}\) transformants were selected (15, 20). Standard large scale 3l cultures are grown in Bello Supernova Flasks™ with magnetic stirring and temperature maintained at 25 °C.

**Membrane Preparations**—Cells were broken with glass beads, and membranes were prepared as described previously (20), except that the membranes were incubated for 25 min on ice with 2 μm urea and 100 μM KC\(_{1}\) prior to the final high speed centrifugation. This urea wash removes about 50% of the membrane-associated protein without affecting the Na\(^+\),K\(^+\)-ATPase activity. Membranes were stored at −80 °C in MOPS-Tris, 10 μM pH 7.2, EDTA, 1 mM, glycerol, 25% with protease inhibitors. Roughly 1 g of membrane protein is obtained per 3 liters of culture. Renal Na\(^+\),K\(^+\)-ATPase was prepared as described in Ref 23.

**Purification of Recombinant Na\(^+\),K\(^+\)-ATPase**—Urea-treated membranes were homogenized well with n-dodecyl-β-maltoside (DDM, 2.1 w/v) in a medium containing NaCl, 250 mM; Tris-Cl, 20 mM, pH 7.4; imidazole, 5 mM; phenylmethylsulfonyl fluoride, 0.5 mM; and glycerol, 10%. The unsolubilized material was removed by centrifugation. The soluble material was incubated for 2–2.5 h at 4 °C with Ni\(^{2+}\)-NTA beads, at a ratio of 1 ml of beads per supernatant from 100 mg of membrane protein. For standard preparations, 1 ml of beads was washed with 10 ml of buffers containing NaCl, 250 mM; Tris-Cl, 20 mM, pH 7.4; DDM; 0.2 mg/ml DOPS (or other combinations of lipid), 0.05 mg/ml glycerol; 10%; and imidazole, 30 mM (first wash) or imidazole, 60 mM (second wash). The Na\(^+\),K\(^+\)-ATPase was eluted by mixing the beads for 30 min on ice with 1 ml of a solution containing imidazole, 250 mM; NaCl, 100 mM; Tris-Cl, 20 mM, pH 7.4; DDM, 0.2 mg/ml; DOPS (or other combinations of lipid), 0.05 mg/ml; glycerol, 40%. The eluted protein was stored at 0 °C. In some cases KC\(_{1}\) replaced NaCl in the elution buffer.

**Deglycosylation**—The recombinant protein (60 μg) in the elution buffer was incubated with Endo-H (2500 units) overnight on ice. Samples were then applied to gels or size exclusion HPLC.

**Size-exclusion HPLC for Apparent M, Determination—DDM-solubilized renal Na\(^+\),K\(^+\)-ATPase or Ni\(^{2+}\)-NTA bead purified recombinant Na\(^+\),K\(^+\)-ATPase (~100 μg of protein) were injected onto a Superdex 200 size-exclusion HPLC column (300 × 10 mm, Amersham Biosciences) using an ETA/6 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; DDM, 0.2 mg/ml; and DOPS, 0.02 mg/ml. Fractions were collected in a fraction collector and analyzed for ATPase activity or applied to gels. Apparent M, values of the eluted Na\(^+\),K\(^+\)-ATPase were calculated from the equation: Elution volume = A\_log M, B, where A = 4.681 ± 0.315 and B = 22.77 ± 0.723, defined by linear regression of data on elution of standard soluble proteins.

**Size-exclusion Chromatography for Stokes Radius Determination and Sedimentation Velocity Experiments**—Analytical SEC-HPLC was performed with System Gold from Beckman instruments. A 0.78–3 cm TosohAas TSK-gel G3000SWx2 column was equilibrated with 2–3 volumes of buffer (150 mM NaCl, 50 mM Na-HEPES (pH 7.4), 0.2 mg/ml DDM, 0.003 mg/ml protein (20 °C)). 100 μl of 5 mg/ml Tes-Tris (pH 7.4), 7% glycerol containing 8 mg of DDM was added to 4 mg of protein from pig kidney membranes in 500 μl of 250 mM sucrose. After 15 min at 0 °C, the sample was centrifuged at 50,000 rpm for 5 min, 4 °C, in a TL-100 rotor, and 50 μl was applied to the column at 1 ml/min. Fractions were collected along the elution profile and stored at 0 °C before sedimentation velocity experiments. Protein standards with Stokes radius (R,) known to be unchanged in the presence of DDM (24) were used to calibrate the volume of the column in the same buffer as above: ferritin (6.3 nm), bovine γ globulin (5.2 nm), chicken ovalbumin (2.8 nm), and equine myoglobin (1.9 nm). Partition coefficients (K,) were determined using K, = (V, – V,)/(V, – V,). Where V, is the elution volume of the proteins. Thigroglubin and vitamin B-12 were used as markers of void volume (V,0) and total volume (V,1), respectively. The calibration standards above R, versus K, was used to interpolate the R, of Na\(^+\),K\(^+\)-ATPase-DDM complex.

**Sedimentation Velocity Experiments**—These were made on two subsequent fractions at 1.04 and 0.87 mg/ml of the main peak of the chromatogram or directly after its solubilization followed by 5-min centrifugation at 40,000 rpm from the membranes. The sedimentation profiles were obtained every 5 min at 42,000 rpm and 10 °C for a sector width of 3 cm, using an A40 ultracentrifuge. Sedimentation velocities were calculated from the equation: S, = (V, – V,)/(V, – V,). Where V, is the elution volume of the proteins. Thigroglubin and vitamin B-12 were used as markers of void volume (V,0) and total volume (V,1), respectively. The calibration standards above R, versus K, was used to interpolate the R, of Na\(^+\),K\(^+\)-ATPase-DDM complex.

**Analytical SEC-HPLC was** available at www.analyticalultracentrifugation.com (input values: partial specific volume of 0.76 ml/g, viscosity of 1.35 cp, density of 1.007 ml/g, and frictional ratio of 1.25; the confidence level for the regularization procedure was 0.7), and the interpretation of the S, values was carried out as described recently (26). The ratio of the size exclusion HPLC peak 2 of the pig kidney Na\(^+\),K\(^+\)-ATPase, or to elution buffer without protein, and concentrated by centrifugation on a Centricon 100 ultrafiltration filter. The [1^4]C-DDM in the retentate without protein was subtracted from that with protein, and the excess of bound DDM was calculated per milligram of protein. DDM micelles have a molecular mass of 60–70 KD and the majority of unbound DDM (85%) is not measured.

**Kidney Lipids**—The kidney lipids were extracted from pig renal cortex membranes with chloroform/methanol, and the extract was dried and redissolved in chloroform (see Ref. 29).

**SDS-PAGE and Western Blots**—12–25 μg of yeast membranes was mixed with 5-fold concentrated sample buffer containing phenylmethylsulfonyl fluoride (1 mM) and separated on 10% Tricine SDS-PAGE (27). Gels were stained with Coomassie, scanned with an imaging densitometer (GS-690, Bio-Rad), and analyzed using the Multi-analyst software (Bio-Rad). Immunobots were blotted with anti-KETTY antibodies, which recognize the C terminus of the α subunit, with or without β antibodies raised against the extracellular domain of the β subunit (28).

**Biochemical Assays—Oubain binding to membranes was assayed using ['H]Oubain essentially as described in Ref. 19. Na\(^+\) K\(^+\)-ATPase activities were assayed at 0 °C using [γ-32P]ATP, routinely at 2 μM final.
concentration (see Refs. 19 and 20) or at 37 °C at 1 mM ATP. Usually 1 µl of recombinant enzyme (~0.2–0.4 µg of protein) was added to 100 µl of the reaction medium. Initially, DDM and lipid, at concentrations equal to that in the elution buffer, were added to the reaction media, but later it was found that they can be omitted. For measurement of the Km for ATP at 37 °C the concentrations were varied from 5 to 500 µM and data were fitted to a hyperbolic function using Enzfitter.

Mass Spectrometry—The β subunits bands identified on un-fixed gels after staining with Gelcode, were electroeluted with 0.05% SDS, and the SDS was replaced with 1% deoxycholate prior to MALDI-TOF mass spectrometry analysis (described in detail in Ref. 30).

RESULTS

The clones α/β-his-, his-α/β, and his-α/his-β were transfected into the strain P. pastoris and His” and mut5 clones were selected, cells were grown, membranes were prepared, and expression of the α subunit was examined by immunoblots (see Ref. 20). The α subunit was not expressed in the his-α/β or his-α/his-β clones, but was expressed well in α/β-his clones. Different α/β-his clones showed wide variations in α subunit levels (20). The clone expressing the highest level of α subunit was selected and grown up in large spinner bottles. Table I presents ouabain binding capacity, Na+/-K+-ATPase activity, and calculated turn-over numbers for representative membrane preparations expressing α/β (without a His tag) or α/β-his subunits. The values are quite similar, and the turn-over numbers are close to expected values for renal Na+,K+-ATPase (23). Thus, the His tag did not significantly affect expression levels or activity.

Of three non-ionic detergents, C12E10, Fos-choline-12, and DDM, compared for efficiency of solubilization of the yeast membranes and preservation of Na+,K+-ATPase activity, DDM was found to be the optimal detergent. In a medium containing 250 mM NaCl, 2 mg/ml DDM, and 1 mg/ml membrane protein, ~70% of the Na+,K+-ATPase was dissolved and activity was preserved. All the detergent-protein ratios higher than 2:1 w/w, Na+,K+-ATPase was progressively inactivated, although the activity could be preserved by adding soya bean phospholipid (e.g. 0.5 mg/ml lipid with 5 mg/ml DDM). The latter finding provided the first indication for a lipid requirement, but solubilization was done routinely at 2 mg/ml DDM without added lipid, which was found to interfere with binding of the protein to Ni2+-NTA beads.

Table I: Purification of Na+,K+-ATPase activity, ouabain binding, and turnover numbers of membrane-bound recombinant porcine Na+,K+-ATPase (α/β1 or α/hisβ1).

| Ouabain binding | Na+,K+-ATPase | Turnover number |
|-----------------|---------------|----------------|
| pmoles/mg protein | µmoles/min/mg protein | min⁻¹ |
| α/β1 | 30.25 | 0.228 | 7536 |
| α/hisβ1 | 23.4 | 0.17 | 7272 |

In elution buffers containing DDM without added phospholipids, the α/β-his-β complex has no Na+,K+-ATPase activity. The finding that added soya bean phospholipid preserves ATPase activity of membranes dissolved at a higher DDM concentration suggested that lipid may be required. It has also been reported that addition of phosphatidylserine maintains activity of C12E10-solubilized Na+,K+-ATPase (31). To test the effects of phospholipids, synthetic DOPC, a neutral phospholipid, or synthetic DOPS, an acid phospholipid, or mixtures of DOPC and DOPS, were added to the washing and elution buffers, and their ability to preserve Na+,K+-ATPase during storage at 0 °C was compared (Fig. 2). Previously we have observed instability and cleavage of the α subunit in yeast membranes prepared in K⁩-containing media. Also solubilization of the membranes with DDM in 250 mM KCl rather than NaCl does not preserve the activity well (not shown). Thus, the effects of the added phospholipids were also compared for media containing either 100 mM NaCl or 100 mM KCl, conditions that should stabilize either Ei,Na or Ei,K conformations, respectively (1, 9). The result in Fig. 2 was both surprising and highly informative. Protein eluted with DOPC in a Na+-containing medium shows Na+,K+-ATPase activity at 0 °C, and activity is maintained constant for about 2 weeks. When eluted with DOPC in the K⁩-containing medium the initial activity was lower, and it was lost within 2 days. By contrast when the protein was eluted with DOPS or mixtures of DOPC/DOPS the initial Na+,K+-ATPase activity was higher, and there was no significant difference between the Na+-containing and K⁩-containing media. During storage over 2 weeks the mixture DOPC/DOPS preserved activity somewhat better than DOPS alone.

However, the more remarkable observation is that, although either DOPC or DOPS maintained activity in the sodium-containing medium, only the DOPS stabilized the protein against inactivation in the K⁩-containing medium. The protein in the experiment of Fig. 2 was eluted in the presence of 1 mg/ml DDM plus 0.5 mg/ml lipid, but it was found subsequently that 0.2 mg/ml DDM plus 0.05 mg/ml phospholipids sufficed. The results in Fig. 2 provide one indication for a direct interaction of the recombinant protein with DOPS (see “Discussion”).

As an initial indicator of the functional state of the eluted recombinant enzyme, its Na+,K+-ATPase activity was compared with activities of membrane-bound or DDM-solubilized pig kidney Na+,K+-ATPase at 0 °C. The specific Na+,K+-ATPase activity of the recombinant protein at 0 °C, in the range 25–40 nmol/min, was distinctly higher than that of either the membrane-bound (9 nmol/min) or DDM-solubilized pig kidney Na+,K+-ATPase (12 nmol/min), of comparable purity (~50%). The apparent paradox of a more active recombinant protein has been resolved by a finding that the

![FIG. 1. Purification of recombinant Na+,K+-ATPase by Ni2+-NTA chromatography. Each lane was loaded with about 10 µg of protein. Ub, unbound fraction of DDM solubilized membranes; PKE, pig kidney enzyme.](image-url)

2 D. Strugatsky and S. J. D. Karlish, unpublished data.
difference was due to non-native lipids. When lipids extracted from pig kidney were used for the washing and elution of the recombinant protein, a much lower activity was observed, 11–13 nmol/min/mg, which is in the same range as the membrane-bound or detergent-solubilized native renal Na⁺,K⁺-ATPase. This effect of kidney lipids could be related to the presence of cholesterol, because preliminary experiments have shown that washing the beads and elution of recombinant protein with 30% cholesterol added to the DOPC/DOPS mixture inhibits the ATPase activity at 0 °C by 2- to 3-fold (not shown). Overall, and within the accuracy of the data, the recombinant enzyme can be considered at least as active as the soluble Na⁺,K⁺-ATPase at 0 °C. In these initial experiments the Na⁺,K⁺-ATPase activity was always measured at 0 °C at 2 μM ATP, with detergent and lipid added to the reaction medium at the same concentrations as used in the Ni-NTA beads elution buffer. Subsequently, however, it was found that lipid and detergent could be omitted and indeed Na⁺,K⁺-ATPase activity could also be measured conveniently at 37 °C. Fig. 3 shows a time course of ATP hydrolysis by the recombinant enzyme at 37 °C without and with ouabain, 1 mM. There are two important features. First, the ATP hydrolysis is linear for at least 10 min, in a medium in which the detergent and DOPS are diluted by 100-fold to 0.002 mg/ml and 0.0005 mg/ml, respectively. An implication is that the protein is stable in these conditions, and DOPS is bound to the protein (see “Discussion”). Second, the activity is fully inhibited by 1 mM ouabain, as expected for purified Na⁺,K⁺-ATPase.

For further purification and comparison of molecular size and activities, in the same detergent and phospholipid environment, we have separated both DDM-solubilized renal Na⁺,K⁺-ATPase and purified recombinant enzyme by size exclusion HPLC (Fig. 4). The HPLC profiles (upper) and gels (lower) show that both renal and recombinant enzymes in DDM elute at equivalent volumes, corresponding to peak 2, whereas the other peaks contain either no proteins or only contaminant proteins. The HPLC profile run without added DOPS was nearly the same as in Fig. 4, but the eluted protein was inactive. In a re-chromatograph of peak 2 of renal Na⁺,K⁺-ATPase after concentration on a Centricon 100 ultrafiltration device (Fig. 5), the protein eluted at exactly the same volume. The same was true after dilution of the protein about 10-fold or after re-chromatographing the protein in peak 2 in an elution buffer containing a very high DDM concentration (15 mg/ml). The results of re-chromatography show that the molecular species in peak 2 is a stable species. As judged by scanning densitometry of stained gels taken from experiments like that in Fig. 4, the purity of both the renal and recombinant Na⁺,K⁺-ATPase (peak 2) after HPLC was in the range 70–80%.

Calibrated size exclusion chromatography with standard soluble proteins provided apparent molecular mass of the renal and recombinant proteins of 468 ± 27 kDa, Rₑₑ = 6.5 nm, and 450 ± 25 kDa, respectively, corresponding to complexes, including bound detergent and lipids. Two separate measurements of bound DDM, by ultrafiltration of renal Na⁺,K⁺-ATPase with bound [¹⁴C]DDM (see “Experimental Procedures”), gave estimates of 0.48 and 0.52 mg bound DDM per mg of protein, respectively. Sedimentation velocity experiments show that Na⁺,K⁺-ATPase is in a quite homogeneous form, particularly the top fraction of peak 2 from size exclusion chromatography (Fig. 6, A–C). The data were analyzed nicely in terms of a distribution of sedimentation coefficients, which showed, at 278 nm, for two subsequent tubes in the chromatography, a major peak at nearly the same position: sₑₑ,w = 10.05 and 10.29 S. In addition to this main peak, the c(s) analysis showed minor contributions of other species (Fig. 6C). Logically, the first and second fraction from the chromatography contained more of the

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**Fig. 2.** Na⁺,K⁺-ATPase activity of recombinant enzyme prepared with DOPC, DOPS, or DOPC/DOPS. The six different enzyme preparations, eluted from Ni²⁺-NTA beads, were stored at 0 °C. At the indicated times, over 11 days, aliquots corresponding to 0.3 μg of protein were transferred to the reaction medium for measurement of Na⁺,K⁺-ATPase activity at 0 °C. The ratio of DOPC/DOPS was 2:1 (w/w).

**Fig. 3.** Time course of ATP hydrolysis at 37 °C and inhibition by ouabain. 5 μl of the recombinant enzyme (~1.5 μg) was added to 500 μl of the standard Na⁺,K⁺-ATPase reaction medium, containing 1 mM ATP plus [γ-³²P]ATP, without or with 1 mM ouabain, but no added lipid or detergent. At the indicated times aliquots were removed for analysis of ³²P, released.

**Fig. 4.** Purification of renal and recombinant Na⁺,K⁺-ATPase by size exclusion HPLC. Pig kidney Na⁺,K⁺-ATPase was dissolved in DDM (2 mg/mg of enzyme), the insoluble material was removed by centrifugation, and ~100 μg of the supernatant protein was injected into the column. Similarly about 100 μg of the recombinant enzyme eluted from Ni²⁺-NTA beads was injected (upper). The marked peaks were collected and aliquots applied to the gel (lower).
Purification of Na\(^{+}\),K\(^{+}\)-ATPase Expressed in P. pastoris

The partial specific volume of the glycosylated αβ complex (molecular mass = 161.3 kDa) is 0.729 ml/g, that of DDM 0.824 ml/g, that of lipid 0.93 ml/g given for phosphatidylserine (32). Δ\(\text{DDM}\) and Δ\(\text{lp}\) are the amounts in grams per gram of nonglycosylated protein, DDM, and lipid. The Stokes radius, \(R_s\), was calculated with a frictional ratio of 1.25.

Table II presents the calculation of the Stokes radius, the sedimentation coefficient, the buoyant molecular weight, and the molecular weight for a globular compact complex with different hypotheses of bound detergent and lipid. The experimental value of \(\text{Rs} = 10.14\) S, combined with the experimental hydrodynamic radius of 6.5 nm from gel filtration, would correspond to a buoyant molecular mass of 75 kDa, i.e. compatible with a monomer solvated by a large amount of detergent or lipids. In conclusion, from sedimentation velocity

larger and lighter species, respectively. The effect of the concentration dependence was evaluated using the protein freshly solubilized from the membrane. The c(s) profiles are qualitatively similar to those obtained after chromatography (data not shown). The \(s_{20,w}\) values decreased when the concentration was increased (Fig. 6D), which can be related to an under-estimated viscosity or to excluded volume effects. In the case of equilibrium association, the opposite effect would be expected. The linear extrapolation of \(s_{20,w}\) to infinite dilution was 10.07 S, i.e. the same values as after chromatography. To summarize, these results indicate that the protein is essentially homogeneous with \(s_{20, w} = 10.14 \pm 0.13\) S; the species purified by chromatography did not differ from that obtained directly from the solubilized membrane; the results do not provide any indication for an auto-association equilibrium.

The data obtained with interference optics show essentially the same feature (not shown), but the relative intensities of the peaks, as measured by the number of fringe \(J\), are different from the c(s) built in absorbance units, A, at 278 nm. The contributions at lower \(s-s_{20, w}\) values in the range 5–8 S (Fig. 6C, left part) had a very large \(J/A\) value of 21.0 ± 2.7, suggesting a very high detergent plus lipid/protein ratio. Their concentration was larger for the fractions eluted later in the chromatography or for samples from the membrane that were more diluted in DDM solvent. The \(s\)-value was larger than expected for simple DDM micelle (\(s_{20,w}\) of 3 S). These two features suggest that these species correspond to complexes of detergent and peptides or lipids and that they are dissociated by the dilution of the membrane in the detergent and can be separated by chromatography. These species could be in equilibrium with detergent micelles (because the latter were not seen in the c(s) analysis). For the main species at 10.14 S (panel C), the mean value was \(J/A = 5.4 \pm 0.6\), with slightly larger values of \(J/A = 6\) for the sample from chromatography, and smaller values of \(J/A = 5\) for the protein from the membrane. The contribution of the nude glycosylated protein was \(J/A = 3.0\) and not for a dimer (15.9 ± 0.9 S). The experimental value of \(s_{20,w}\) (10.14 ± 0.13 S) is compatible with the value calculated for a monomer (10.0 ± 0.5 S), and not for a dimer (15.9 ± 0.9 S).

Table II presents the calculation of the Stokes radius, the sedimentation coefficient, the buoyant molecular weight, and the molecular weight for a globular compact complex with different hypotheses of bound detergent and lipid. The experimental value of \(\text{Rs} = 10.14\) S, combined with the experimental hydrodynamic radius of 6.5 nm from gel filtration, would correspond to a buoyant molecular mass of 75 kDa, i.e. compatible with a monomer solvated by a large amount of detergent or lipids. In conclusion, from sedimentation velocity

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\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Hypothesis} & \text{R}_s & \text{s}_{20,w} & M_b & \text{R}_s & \text{s}_{20,w} & M_b \\
\hline
\text{Monomer} & \text{nm} & \text{S} & \text{kJdA} & \text{nm} & \text{S} & \text{kJdA} \\
\hline
\text{Dimer} & \text{nm} & \text{S} & \text{kJdA} & \text{nm} & \text{S} & \text{kJdA} \\
\hline
\Delta \text{DDM} = 0.5 & 5.2 & 9.8 & 56.9 & 6.6 & 15.6 & 113.8 \\
\hline
\Delta \text{DDM} = 0.5 & 6.3 & 9.6 & 66.4 & 7.9 & 15.3 & 132.8 \\
\hline
\Delta \text{lp} = 0.9 & 6.2 & 10.6 & 72.6 & 7.8 & 16.9 & 145.2 \\
\hline
\end{array}
\]

3 The buoyant molecular weight (\(M_b\) in Table III) corresponds to the experimentally determined parameter in these experiments: \(M_b = M(1 - \psi_0)\), where \(M\) is the molecular weight, \(\rho\) is the solution density, and \(\psi_0\) is the buoyant density of M. For membrane proteins, to take into account the contribution of bound detergent and remaining bound lipid, it is often replaced by the formally similar expression \(M_b = M(1 - \Phi P)\), where \(M\) is the protein molecular mass (without bound detergent and lipid) and (1 – \(\Phi P\)) is an effective buoyant density term (32).

Fig. 5. Re-chromatograph of the HPLC peak of renal Na\(^{+}\),K\(^{+}\)-ATPase. Peak 2 of the first run of DDM-solubilized pig kidney Na\(^{+}\),K\(^{+}\)-ATPase was collected and concentrated prior to re-injection.

Fig. 6. Sedimentation velocity analysis of renal Na\(^{+}\),K\(^{+}\)-ATPase. A, experimental and fitted sedimentation profiles obtained at 10 °C and 42,000 rpm for pig kidney Na\(^{+}\),K\(^{+}\)-ATPase from size exclusion chromatography in the presence of 0.2 mg/ml DDM. B, difference between the experimental and fitted curves. C, c(s) analysis for two successive fractions (solid line, data from panels A and B; dashed lines, sample freshly diluted from solubilized Na\(^{+}\),K\(^{+}\)-ATPase). D, concentration dependence of \(s_{20,w}\) for samples freshly diluted from solubilized Na\(^{+}\),K\(^{+}\)-ATPase; the viscosity of the solutions was estimated using tabulated data.
experiments, the most probable result would be a monomer of glycosylated αβ complex.

The Na⁺,K⁺-ATPase activities at 37 °C of the HPLC-purified renal and recombinant enzyme have been compared (Table III). The specific activities of the HPLC-purified recombinant and pig kidney enzymes, in the range 9–15 μmol/mg/min, were similar to each other, but are significantly lower than the rate of the membrane-bound pig kidney Na⁺,K⁺-ATPase. Also, the purest preparations of pig kidney Na⁺,K⁺-ATPase described in the literature have higher activities, in the range 35–40 μmol/mg/min (23). The values for the Kₘ for ATP (column 3), which, again, were similar for the HPLC-purified renal and recombinant enzymes (about 30 μM), were much lower than the Kₘ measured for membrane-bound renal Na⁺,K⁺-ATPase (200–400 μM), determined previously. This suggests that the renal and recombinant enzymes are stabilized in E₁, or E₃-P conformations, by comparison with the membrane-bound renal Na⁺,K⁺-ATPase, and provides a clue to the origin of the lower specific activities (see "Discussion").

Figs. 7 and 8 and Table IV provide evidence for deglycosylation of the recombinant β subunit by Endo-H and an additional indication for an interaction of DOPS with the enzyme. As seen in Fig. 7 incubation of purified recombinant enzyme overnight with Endo-H at 0 °C converted the two glycosylated bands (β1 and β2, lane C) to a single sharp band (βD, lane Endo-H). Endo-H itself is also seen in lane 2. After further purification of the control and deglycosylated enzyme by HPLC (as in Fig. 4, upper), peak 2 contained the α and β subunits, separated from Endo-H, and only minor contaminating proteins. Masses of β1, β2, and βD as well as βR from renal Na⁺,K⁺-ATPase, estimated by MALDI-TOF mass spectrometry (30), are given in Table IV. The mass of βD is close to the predicted protein mass of the deglycosylated β subunit plus the His tag. Fig. 8 shows results of Na⁺,K⁺-ATPase measurements of the recombinant enzyme, prepared either with DOPC or with DOPS, with Na⁺ or K⁺, without or with incubation with Endo-H. The striking finding is that deglycosylation largely inactivates Na⁺,K⁺-ATPase prepared with DOPC, whereas that prepared with DOPS is largely intact. As discussed below the conclusion is that DOPS protects against inactivation associated with deglycosylation.

**DISCUSSION**

Purification of the recombinant Na⁺,K⁺-ATPase provides insights into interactions with phospholipids, the oligomeric state of the protein, and role of glycosylation, as well as opening up new experimental possibilities. The requirement for added phospholipids to maintain activity of recombinant or renal Na⁺,K⁺-ATPase shows that the DDM is not a perfect substitute for either the native yeast or kidney membrane lipids. There are several indications that the phospholipids, particularly DOPS, interact directly with the recombinant enzyme. First, only low concentrations of either DOPC or DOPS (about 25 μM) were required for full preservation of activity upon elution from beads. Furthermore, even when the eluted protein was diluted by 100-fold into the Na⁺,K⁺-ATPase reaction medium the activity was maintained for at least 10 min at 37 °C (Fig. 3). Because, in these conditions, the free detergent was present at only 0.002 mg/ml, which was about 40-fold lower than the critical micelle concentration (0.08 mg/ml), it is clear that at the very low ambient lipid concentration (0.0005 mg/ml or 0.25 μM), there was no possibility of lipid dissociation and re-distribution to mixed lipid-detergent micelles. Thus, the maintenance of Na⁺,K⁺-ATPase activity indicates that the DOPS must still be associated with the protein. Second, the Na⁺-or K⁺-dependent difference between DOPS and DOPC in preserving the Na⁺,K⁺-ATPase activity of the recombinant protein (Fig. 2) provides evidence that the conformation, E₁Na or E₂(K), determines the selectivity of a direct interac-

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**TABLE III**

*Specific Na⁺,K⁺-ATPase activities at 37 °C and Kₘ for ATP of HPLC-purified recombinant and renal Na⁺,K⁺-ATPase*

| Enzyme Type                  | Na⁺,K⁺-ATPase activity | Kₘ ATP |
|------------------------------|------------------------|--------|
| HPLC-purified recombinant    | 11.7 ± 1.52            | 29.0 ± 10 |
| HPLC-purified renal          | 15.1 ± 0.35            | 42.9 ± 3.35 |
| Pig kidney Na⁺,K⁺-ATPase     | 8.7 ± 1.1 (n=3)        | 33.5 ± 2.8 |

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**Fig. 7. Deglycosylation of recombinant Na⁺,K⁺-ATPase.** Recombinant Na⁺,K⁺-ATPase was treated with Endo-H as described under "Experimental Procedures." Aliquots of control and deglycosylated enzyme were applied to the HPLC column, and fractions 2 were collected. Renal Na⁺,K⁺-ATPase was also injected, and fraction 2 was collected. C, control; βR, renal Na⁺,K⁺-ATPase; β1 and β2, recombinant; βD, deglycosylated recombinant Na⁺,K⁺-ATPase.

**Fig. 8. Inactivation of Na⁺,K⁺-ATPase activity associated with deglycosylation in DOPC, and protection against inactivation by DOPS.** Recombinant Na⁺,K⁺-ATPase prepared with either DOPC or DOPS in media containing either 100 mM NaCl or KCl was deglycosylated overnight with Endo-H, the samples were applied to the HPLC column, and aliquots of peak 2 were taken for measurement of Na⁺,K⁺-ATPase activity.
tion, at least of DOPS. In reality, because either DOPC or DOPS (somewhat better) preserve activity in the Na⁺-medium, but only DOPS preserves activity in the K⁺ medium, there may be at least two sets of phospholipid-protein interactions. One interaction in the E₁,Na conformation could be satisfied by either DOPC or DOPS, whereas the other interaction in the E₂(K) conformation was preserved by DOPS and not by DOPC (or perhaps DOPC interacts but inhibits). Third, the difference in Na⁺,K⁺-ATPase activity after deglycosylation of recombinant enzyme prepared with DOPS or DOPC, respectively, was also highly indicative of a direct effect of DOPS to stabilize α/β interactions, as discussed below.

Previous work with Na⁺,K⁺-ATPase, in reconstituted liposomes, or lipid-enriched or -depleted native membranes, revealed effects of different phospholipids classes (35, 36), or fatty acid chain length and saturation (37, 38), or cholesterol (39–41). A recent study demonstrated an important role for “hydrophobic matching” of lipid and protein trans-membrane segments (36). Although selective effects of lipid class, chain length, or saturation may indicate direct phospholipid- or cholesterol-protein interactions (as proposed in Refs. 39 and 40), in a bilayer membrane, bulk physical properties, such as fluidity and bilayer width, and the ensuing variation in “hydrophobic matching”, could constrain the protein structure and affect function. Thus, no firm conclusions on direct lipid-protein interactions are possible. By contrast, the uncertainty is removed with the detergent-solubilized purified recombinant Na⁺,K⁺-ATPase. A report that added phosphatidylycerine maintains activity of C₈E₈-solubilized renal Na⁺,K⁺-ATPase (31), is consistent with a direct phosphatidylycerine-protein interaction, although that conclusion was not emphasized, because the study was concerned primarily with the oligomeric state of the protein. Bound lipids have been observed in crystal structures of several membrane proteins (42). Also, addition of synthetic DOPC to the detergent-solubilized cytochrome b₅₆₂ complex from photosynthetic membranes dramatically improves crystallization (43). Thus, evidence is accumulating for specific lipid-protein interactions, and a concept is emerging that lipids are integral structural components of various membrane proteins (42).

The Na⁺,K⁺-ATPase expressed in the yeast membranes is less stable than in native pig kidney membranes (20), and in K⁺-containing media it is cleaved by endogenous proteases. With hindsight one could hypothesize that sub-optimal lipid-protein interactions are the source of instability.

The evidence for an interaction with DOPS, and possibly DOPC, does not exclude the possibility of additional interactions with other lipids, such as cholesterol, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, sphingolipids, or effects of chain length and bond saturation, etc. It would be important to know if other acid phospholipids have similar effects to DOPS. A preliminary result with cholesterol is mentioned above. Clearly a systematic study of lipid effects is warranted, utilizing the advantage of the purified recombinant Na⁺,K⁺-ATPase, for looking at lipid-protein interactions.

The specific Na⁺,K⁺-ATPase activities of purified recombinant and renal enzymes are similar to that of the membrane-bound renal Na⁺,K⁺-ATPase at 0 °C. However, at 37°C, the activities of HPLC-purified recombinant and renal enzymes are significantly lower than that of membrane-bound renal Na⁺,K⁺-ATPase (Table III), even if one takes into account that they are only 70–80% pure. It is not yet excluded that a fraction of both recombinant and renal enzyme is inactive, but an alternative explanation is suggested by the low values of $K_m$ for ATP (Table III), for these imply that both proteins are stabilized in $E_1$ or $E_1\cdot P$ conformations compared with the membrane-bound renal Na⁺,K⁺-ATPase. In this case, they are expected to have lower activities. Phosphorylation assays have shown directly that about half of the phosphoenzyme generated with sodium, magnesium, and ATP, is ADP-sensitive for either soluble recombinant or renal Na⁺,K⁺-ATPase. This indicates that the $E_1\cdot P$ conformation is indeed stabilized relative to $E_2\cdot P$. In addition, preliminary fluorescence data with a fluorescein-labeled recombinant protein (see Ref. 44) shows directly that the $E_1$ conformation is stabilized relative to $E_2$. Overall one could hypothesize that, although DOPS in DDM maintain activity, additional lipid-protein interactions may be required to fully mimic the native renal membrane environment.

The sizes of renal and recombinant Na⁺,K⁺-ATPase are very similar as judged by size-exclusion HPLC. Combined size-exclusion HPLC and sedimentation velocity on renal Na⁺,K⁺-ATPase suggest that it is a (α/β) monomer binding a significant amount of detergent and lipid: a total of about 1.4 g/g, consistent with the value estimated for DDM alone of 0.5 g/g.

The predicted masses of the α and β monomers are 161 kDa (renal) and 156 kDa (recombinant), respectively, consisting of $\alpha$, 112.3 kDa; $\beta$, including sugars, measured by mass spectrometry, renal, 49 kDa; recombinant, average 43.5 kDa (see Table IV). With 1.4 g/g bound detergent or lipid, the masses of the renal complex would reach 367 kDa, the Stokes radius for a compact complex would be 6.2 nm, to be compared with the apparent molecular mass value from HPLC of 468 kDa or Stokes radius of 6.5 nm. Contrary to polyoxyethylene detergents (45), DDM is not known to overestimate Stokes radii of membrane proteins (24, 46). Our difference can easily be accounted for by a shape effect (due to elongated shape and/or glycosylation).

Judging by the HPLC profiles in Figs. 4 and 5 and sedimentation velocity experiments in Fig. 6, there was little evidence for higher order oligomers except in the void volume. Furthermore, because peak 2 of the renal enzyme appeared in the same position when re-chromatographed (Fig. 5), even when diluted 10-fold, or when run in DDM at 15 mg/ml, the monomeric species in DDM is stable, and does not appear to be in a mobile equilibrium with other molecular species. Our findings are not inconsistent with recent work, showing by immunoprecipitation that Na⁺,K⁺-ATPase expressed in baculovirus-infected insect cell membranes, and dissolved in DDM, can exist as oligomers of the α/β heterodimer, if we consider the oligomers detected in the void volume by HPLC, the presence of minor heavier species in addition to monomer in the sedimentation profiles, and the tendency of the protein to slowly auto-associating.

4 Y. Lifshitz, R. Goldshleger, H. Garty, and S. J. D. Karlish, unpublished observation.

5 T. Belogus and S. J. D. Karlish, unpublished observation.

| Mass, kDa ± S.E. | $\beta_1$ | $\beta_2$ | $\beta_3$ |
|-----------------|----------|----------|----------|
| 48,994 ± 256    | 44,386 ± 271 | 42,644 ± 9.6 | 38,396 ± 164 |
| $n = 10$        | $n = 3$  | $n = 4$  | $n = 3$  |
| 34,967 protein  | 14,027 ± 256 | 5,990 ± 316 | 4,248 ± 165 |
| Estimated mass of carbohydrate | 37,816 predicted |
ate with time (data not shown) (47). Of course, the characterization of Na\(^{+}\),K\(^{+}\)-ATPase as a monomeric species in DDM does not preclude that other oligomeric species exist in the membrane. The latter scenario may be favored by work showing that the renal Na\(^{+}\),K\(^{+}\)-ATPase, dissolved in C\(_{12}E_8\), exists as a mixture of monomers, dimers and tetramers, the relative proportion of which are affected by the ligand conditions (48). One could infer that DDM and C\(_{12}E_8\) interact differently with the protein and stabilize different oligomeric structures. Neither does the monomeric organization in DDM bear directly on the controversial issue of which oligomeric structure is required to explain complex features of ligand binding nor the kinetic mechanism (reviewed in Ref. 49). In agreement with our work, the \(\alpha/\beta\) monomer of renal Na\(^{+}\),K\(^{+}\)-ATPase dissolved in C\(_{12}E_8\) was shown to be fully active, i.e. the \(\alpha/\beta\) monomer is the minimal functional unit (50, 51). In any case, the fact that the protein is a stable, mono-dispersive species in DDM solution is potentially important for future structural work.

Deglycosylation of the recombinant \(\beta\) subunit was attempted, because a homogenous deglycosylated protein could be more amenable to crystallization than native renal Na\(^{+}\),K\(^{+}\)-ATPase, the \(\beta\) subunit of which is heterogeneous and is not readily deglycosylated. Whether this is so remains to be seen, but, independently, deglycosylation provided insights into a DOPS-protein interaction and the role of the \(\beta\) subunit.

The susceptibility of the recombinant \(\beta\) subunit to Endo-H indicates that its oligosaccharide is of the Man\(_2\)-GlcNAc\(_2\) type, typical of yeast N-linked glycosylation. The estimated masses of the oligosaccharide chain for \(\beta1\) and \(\beta2\) (44.39 and 42.66 kDa) are consistent with Man\(_{12}\)-GlcNAc\(_2\) or Man\(_n\)-GlcNAc\(_n\), in line with the light glycosylation pattern expected in \(P\). pastoris, as compared with that in other yeasts or higher eukaryotic organisms (52). The \(\beta1\) and \(\beta2\) subunits could be derived from pumps located in the cell membrane (Man\(_{12}\)-GlcNAc\(_2\)) or endoplasmic reticulum (Man\(_n\)-GlcNAc\(_n\)), respectively.

The \(\beta\) subunit is required for correct folding and integration of the \(\alpha\) subunit into the membrane of the endoplasmic reticulum and passage to the cell membrane in a functional form (reviewed in Ref. 9). Glycosylation of the \(\beta\) subunit is thought to stabilize the interaction between \(\alpha\) and \(\beta\) subunits in the endoplasmic reticulum, because deglycosylation reduces the yield of Na\(^{+}\),K\(^{+}\)-ATPase reaching the cell membrane, although the function of those pumps that are expressed is normal (53). Formally, the result in Fig. 8 could be taken to show either that DOPS, but not DOPC, protects against inactivation associated with deglycosylation, or that deglycosylation itself has no effect, whereas DOPC, but not DOPS, inhibits the deglycosylated enzyme. The prior evidence for destabilization of the pump by deglycosylation (53) allows one to exclude the latter explanation. Thus, one can infer that an optimal lipid interaction with DOPS compensates for the destabilizing effect of the deglycosylation, and stabilization by glycosylation and DOPC can be considered complementary. During folding and integration of the \(\alpha/\beta\) complexes into the endoplasmic reticulum membrane, glycosylation of \(\beta\) could stabilize the protein significantly, compensating for sub-optimal lipid interactions due to incomplete embedding of trans-membrane segments. Conversely, when the pump is already in the cell membrane in its optimal lipid environment, glycosylation may no longer be a significant stabilizing factor.

Protection by DOPS against both inactivation associated with deglycosylation and inactivation in a K\(^{+}\) medium could imply, most simply, that the same interaction is involved. Prior knowledge on \(\alpha/\beta\) interactions makes this notion even more attractive. In addition to a chaperone function, the \(\beta\) subunit also affects functional properties of the pump, especially the affinity for K\(^{+}\) activation at the extracellular surface (9). A key site of interaction of \(\alpha\) and \(\beta\) subunits is in the extracellular loop L7/8 of the \(\alpha\) subunit, including the sequence SYGQ, just outside the entrance to M8 (54), which is crucial for the correct assembly of the \(\alpha/\beta\) heterodimer (55). Cross-linking shows that the trans-membrane segment of \(\beta\) is close to M8 of \(\alpha\) (56), and M8 is known to be directly involved in K\(^{+}\) occlusion via Asp-926 (M7). Reduction of S-S bridges in \(\beta\) (57, 58), which probably affects the L7/8-\(\beta\) interaction, or copper-catalyzed oxidative cleavage in L7/8 and \(\beta\) (59), inactivates Na\(^{+}\),K\(^{+}\)-ATPase activity and Rb\(^{+}\) (K\(^{+}\)) occlusion. Thus, perturbations of the L7/8-\(\beta\) interactions could affect the native disposition of M8 (or M7) and hence inactivate Rb\(^{+}\) (K\(^{+}\)) occlusion and Na\(^{+}\),K\(^{+}\)-ATPase activity. For the recombinant protein one could propose that a stabilizing interaction of DOPS with M8, or proximal trans-membrane segments, counter a tendency of either occluded K\(^{+}\) ions, or perturbation of L7/8-\(\beta\) interactions caused by deglycosylation, to destabilize M8 and thus inactivate K\(^{+}\) occlusion.

It has been proposed that, in the endoplasmic reticulum, the \(\beta\) subunit of the K\(^{+}\)-transporting pumps Na\(^{+}\),K\(^{+}\)-ATPase or H\(^{+}\),K\(^{+}\)-ATPase is required for correct folding and membrane integration of trans-membrane segments associated with K\(^{+}\) transport (9). The present findings add the notion that, even in the mature complex, \(\alpha/\beta\) interactions may be required to compensate for the tendency of occluded K\(^{+}\) ions to destabilize the trans-membrane segments and are effective only in the context of the correct phospholipid-protein interaction. The difference from other ion pumps such as Ca\(^{2+}\)-ATPase or H\(^{+}\)-ATPases, as well as a rationale for the lack of a \(\beta\) subunit in those pumps, could be that only K\(^{+}\) ions are intrinsically destabilizing, due to the greater ionic radius (e.g. K\(^{+}\), 1.35 Å compared with Ca\(^{2+}\), 0.9 Å).

The recombinant Na\(^{+}\),K\(^{+}\)-ATPase can be prepared relatively pure, functional, mono-disperse, and deglycosylated in 0.1- to 1-mg quantities. With further purification, and more complete functional characterization to establish that the protein is fully active, crystallization trials may be undertaken. The purified recombinant enzyme should also be useful for advanced structure-function studies using a variety of spectroscopic and biophysical techniques. Purification of \(\alpha/\beta\)FXYD protein complexes, and expression and purification of isoforms of Na\(^{+}\),K\(^{+}\)-ATPase, are other applications now being undertaken.

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