Expression in High Yield of Pig α1β1 Na,K-ATPase and Inactive Mutants D369N and D807N in Saccharomyces cerevisiae*

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Studies of structure-function relationships in Na,K-ATPase require high yield expression of inactive mutations in cells without endogenous Na,K-ATPase activity. In this work we developed a host/vector system for expression of fully active pig Na,K-ATPase as well as the inactive mutations D369N and D807N at high levels in Saccharomyces cerevisiae. The α- and β-subunit cDNAs were inserted into a single 2-μm-based plasmid with a high and regulatable copy number and strong galactose-inducible promoters allowing for stoichiometric alterations of gene dosage. The protease-deficient host strain was engineered to express high levels of GAL4 transactivating protein, thereby causing a 10-fold increase in expression to 32,500 ± 3,000 [3H]ouabain sites/cell. In one bioreactor run 150–200 g of yeast were produced with 54 ± 5 μg of Na,K-pump protein/g of cells. Through purification in membrane bound form the activity of the recombinant Na,K-ATPase was increased to 42–50 pmol/mg of protein. The Na,K dependence of ATP hydrolysis and the molar activity (4,500–7,000 min⁻¹) were close to those of native pig kidney Na,K-ATPase. Mutations to the phosphorylation site (D369N) or prephosphorylation site (D807N) in the α-subunit of Na,K-ATPase, with multiple transmembrane segments, has proven to pose special problems. The Na,K-pump has been expressed in the yeast Saccharomyces cerevisiae (1), COS cells (5), HeLa cells (6), NIH 3T3 cells (7), Xenopus oocytes (8), and in insect cells (9). The properties of isozymes of Na,K-ATPase have been analyzed (10, 11) and important structure-function correlates have been established by work on mutants (5, 7, 12, 13). However, it has been difficult to express Na,K-ATPase in large quantities and higher eucaryotic cell lines like HeLa (6) and COS cells (5) and Xenopus oocytes (8, 14) express endogenous Na,K-ATPase of almost the same magnitude as the transfected activity. The ouabain selection methodology (5, 6) does not allow analysis of the interesting mutants that are blocked in the reaction cycle. The expression level in baculovirus infected insect cells is high, but only a small fraction of the recombinant pumps are enzymatically active, and insect cells also possess endogenous Na,K-ATPase activity (9, 11). A major advantage of yeast cells for expression studies is their lack of endogenous Na,K-ATPase activity. The work of Farley and co-workers (1, 15, 16) demonstrated that yeast cells are capable of expressing fully active Na,K-ATPase at the cell surface, but the expression is limited to levels of 2–4 pmol/mg of protein as determined by [3H]ouabain binding.

In the present work the capacity of S. cerevisiae for the production of enzymatically active Na,K-pumps was characterized with respect to the dependence upon gene copy number, promoter strength, and growth medium composition. The α- and β-subunit gene dosage was altered in parallel by insertion of their cDNAs into a single plasmid with a particularly high and regulatable copy number. The promoter activity was increased by the combination of a strong galactose regulated promoter and a host strain modified to express high levels of GAL4-transactivating protein (17). The plasmid was constructed to allow separation of the growth of the host cells from the phase of Na,K-ATPase expression. Computer-controlled bioreactors were used to increase the yield and growth experiments were performed to examine the influence of medium composition and induction time using [3H]ouabain binding to assess the expression levels. Established methods were modified for partial purification of Na,K-ATPase from the yeast plasma membranes, in conditions where the endogenous H-ATPase was removed. The quantity, ligand binding, and enzymatic properties of the recombinant Na,K-ATPase were determined. The versatility of the expression system was examined by characterization of mutations to two side chains of the α-subunit, the D369N mutation of the phosphorylated side chain (7, 18), and D807N at a presumptive site for cation binding (19). These mutants were devoid of Na,K-ATPase and potassium-dependent para-nitrophenyl phosphatase activities, but they could be expressed in the yeast membranes at the same ω-subunit concentration and [3H]ouabain binding capacity as the wild type Na,K-ATPase. The high yield of Na,K-

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The plasmid pPAP1485 bears the 3.4-kilobase pair EcoRI-GAL10-GAL4 HindIII fragment from pKHin-C inserted into EcoRI and HindIII-digested pUC19. pPAP1488 was constructed by inserting the 850-bp EcoRI-TRP1-EcoRI fragment from pEMBLyex25 into EcoRI-digested pPAP1485.

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to Ho et al. (35). The nucleotide sequences of the two D369N mutagenic primers were 5′- CAT CTG CTC AAA CAA ACC CGG 3′ and 5′- CCG GTT TTT GAG AAT GTG 3′. The mismatched nucleotide changing Asp369 to Asn369 is underlined. A fragment containing the mutation was subsequently cloned into the expression plasmid pPAP1466. The nucleotide sequences of the two D807N mutagenic primers were 5′- GGA ACA GTT GGG CAC AAA CAT GGT TTC C 3′ and 5′- CCG GTT TTT GAG AAT GTG 3′. The mismatched nucleotide changing Asp807 to Asn807 is underlined. A restriction fragment containing the mutation was subsequently cloned into the expression plasmid pPAP1666. The DNA sequences of the entire PCR fragments were confirmed by dideoxy sequencing.

Induction of Na,K-Pump Synthesis—A single colony of transformed yeast cells was selectively propagated until saturation in 10 ml of glucose minimal medium supplemented with leucine (21). Aliquots of 5 ml were subsequently propagated in 100 ml of minimal medium lacking leucine. For small scale production, 10 ml of this preculture were used for inoculation of 3 liters of selective minimal medium containing 0.5% glucose and 2% lactate as carbon source and supplemented with all the other amino acids except leucine, tryptophan, and histidine. Galactose was added to a final concentration of 2% at A600 = 1.0. Routinely, cells were harvested 48 h after addition of galactose.

For large scale production, 100 ml of preculture was used to inoculate 10 liters of minimal medium in an Applicon® fermentor equipped with an ADI 1030 Bio Controller. The culture was agitated at 158 rpm, and air was supplied through a 0.2-μm filter. The carbon source was 0.5% glucose and 2% lactate, and the medium was supplemented with all amino acids except leucine, tryptophan, and histidine. Growth at 30°C in glucose, the pH of the medium was kept at 6.0 by computer-controlled addition of 1 M NaOH. The shift from growth on glucose to growth on lactate was monitored as an increase in pH of the growth medium and a decrease in growth rate. At this point a 10-fold increase in A600 was followed by a final concentration of 2%. Cells were harvested after 48 h.

Isolation of Yeast Membranes—Galactose-induced yeast cells were harvested at 5,800 g for 30 min and washed once in ice-cold water. Yeast cells were resuspended in ice-cold lysis buffer (25 mM imidazole, 1 mM EDTA, 1 mM EGTA, 10% (w/v) sucrose, pH 7.5) containing 1 mM PMSF, 1 μM chymostatin, 1 μM pepstatin, and 1 μM leupeptin. Cells were homogenized in an ice-cold Bead Beater ( Biospec) for three times at 1 min. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was centrifuged at 3,000 × g for 20 min at 4°C. The final supernatant was centrifuged at 100,000 × g for 1.5 h at 4°C. The resulting crude membrane fraction was homogenized in a Teflon-glass Braun homogenizer in ice-cold lysis buffer containing the previously mentioned concentrations of protease inhibitors and kept at 80°C until use.

Gradient Membranes—Crude membranes were fractionated on step gradients consisting of 15 ml of 40% plus 15 ml of 15% (w/v) sucrose in lysis buffer with proteolyis inhibitors. A sample of 6–8 ml containing 40 mg of protein was layered on top, and the gradient was centrifuged for 2 h at 50,000 rpm in the Ti-60 Beckman rotor. The membrane band at the 15/40% interface containing 20–30% of sample protein in 6 ml was collected in a syringe, diluted 5-fold in lysis buffer, and collected by centrifugation at 2 h at 50,000 rpm.

Electrophoretic Analysis and Immunological Techniques—Protein samples were mixed with 2-fold concentrated loading buffer containing 1 mM EDTA, 10% glycerol, and 1% SDS and incubated for 10 min at room temperature, prior to separation in 8% polyacrylamide gels (36). Western blotting was performed according to Kihse-Andersen (37) using a polyclonal anti-α-antibody (anti-KETYY) kindly provided by J. Acke, University of California. For quantitative determinations of Na,K-ATPase proteins, Western blots were digitalized on an HP-Scanjet III P, and densitometry performed using the Cream software from KemEnTech, Copenhagen, Denmark.

Selective SDS Treatment of Crude Yeast Membranes and Purification of Na,K-ATPase—Yeast membranes were treated with SDS as before (38, 39). The final concentration of the solution was 0.64 mg/ml SDS, 2 mg/ml protein, 3.0 mM NaCl, 2.5 mM ImidazoleHCl, 1 mM Na2EDTA, pH 7.5, and 1 mM PMSF, 1 μM chymostatin, 1 μM pepstatin, and 1 μM leupeptin to prevent proteolysis. The SDS-treated membranes were loaded on a step gradient containing two layers of 10 and 37% (w/v) sucrose in the 35-ml tubes of the Beckman Ti-60 angle rotor and

ATPase from yeast and the absence of endogenous activity allowed assays not previously achieved for recombinant enzyme, such as [3H]ATP binding at equilibrium.

Materials and Methods

Strains and Media—S. cerevisiae strains BJ 5457 (α ura3-52 trpl ssp201 hisd201 his32000 pep4::HIS3 prbl11.6R can1 GAL1) (20) and the derivative PAP1500 (α ura3-52 trpl::GAL10-GAL4 lys2-801 leu21 his32000 pep4::HIS3 prbl11.6R can1 GAL1) (this study) were used as host organisms for the expression of the α1 pig Na,K-ATPase. Strain PAP1500 was constructed by integrating BglII-digested pPAP1488 into the trpl locus of BJ 5457 by homologous recombination. Southern blotting of restriction endonuclease digested chromosomal DNA isolated from this strain confirmed the presence of the entire GAL10-GAL4 region in the yeast chromosome (data not shown). Transformation of S. cerevisiae BJ 5457 was achieved using a biotinylated, nick-translated probe prepared as described by Blow and Toft (30). The plasmid pPAP1467 contains the 5.0-kilobase NruI-EcoRI fragment from pGCS3 (31). The 3.4-kilobase XbaI-1-HindIII fragment from pGCS3 was inserted into XbaI-HindIII-digested pEMBLYex4. The resulting polymycin linker region 5′ to the XbaI site was removed by T4 DNA polymerase treatment and ligation after SacI-XbaI digestion. The resulting plasmid was digested with HindIII, Klenow polymerase-treated, and religated to remove the unique HindIII site. A 10-bp HindIII linker (double-stranded deoxy (dGCGAAGCTTCG)) (Boehringer Mannheim) was introduced into the unique NdeI site. The resulting α1 expression plasmid carries the 1.1 pig cDNA under control of the CYC-GAL promoter. The Klenow polymerase-digested 941-bp Ncol-β1-Dral fragment from pNKJ31 was ligated into XhoI-digested, Klenow polymerase-digested RS421. The resulting plasmid carries the Ncol-β1-Dral fragment between the HpaI-1-HATPase promoter and terminator region. The promoter-β1-terminator region present on a HindIII fragment was cloned into the unique HindIII site in pEMBLYex4 in the correct orientation with respect to the CYC-GAL promoter. A 10,637-bp NruI-Hpal fragment was excised from the plasmid to eliminate one of the three KpnI sites. The resulting plasmid was digested with KpnI and religated to remove the PMA1 promoter from the plasmid. The resulting β1 expression plasmid contains the pig β1 cDNA under control of the CYC-GAL promoter. The final α1β1 expression plasmid, pPAP1466, carries the 5.0-kilobase NruI-CYC-GAL-β1-HindIII fragment inserted into the NruI-HindIII-digested α1 expression plasmid.

The plasmid pPAP1647 contains the NheI-URA3-Amp-BglII fragment from pYES2.0 and the NheI-PSA ori-Amp-BglII fragment from pACYC177 (34). The ScaI-PSA ori-ScaI fragment from PAP1647 was cloned into Scal-digested pEMBLYex4. The resulting plasmid pPAP1657 has a 100-fold lower copy number in E. coli compared with pEMBLYex4. The α1 expression plasmid pPAP1666 carries the Apall-1-AattI fragment from pPAP1657, the Apall-1-AattI fragment, and the AattI-β1-AattI fragment from pPAP1466. The resulting α1 expression plasmid has a 100-fold lower copy number in E. coli compared to pPAP1466.
separated by centrifugation for 2 h at 40,000 rpm. A band of 6 ml was collected from the interface of 10 and 37% sucrose through a needle mounted on a syringe, and Na,K-ATPase was collected as a pellet after 3–5 fold dilution and centrifugation at 40,000 rpm overnight.

For ATP binding experiments, gradient membranes were incubated at 2 mg of protein/ml in lysis buffer with 0.3 mg/ml SDS and proteolysis inhibitors for 30 min at 20°C. The mixture was centrifuged for 30 min at 70,000 rpm in the Beckman 100 A ultracentrifuge and resuspended in lysis buffer.

Equilibrium \[^{3H}\] Ouabain Binding to Isolated Membranes—100–200 μg of yeast membrane protein was incubated at 37°C for 1 h in 3 mM MgSO4, 1 mM NaTri5VO4, 1 mM EGTA, 10 mM MOPS-Tris, pH 7.2, in the presence of 10 mM \[^{3H}\] Ouabain (Amersham Corp., specific activity: 36 Ci/mmol) and varying concentrations of cold ouabain, and 1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin to prevent proteolysis. After standing at 4°C for 20 min, bound and unbound ouabain were separated by centrifugation in the Beckman 100 A centrifuge at 70,000 rpm for 30 min at 4°C. Samples were washed twice in ice-cold binding buffer and centrifuged for 10 min at 70,000 rpm. The amount of bound \[^{3H}\] Ouabain was determined by scintillation counting.

Equilibrium ATP Binding—Aliquots containing 10 mg of yeast cell membranes were incubated at 37°C for 1 h in 1 mM sorbitol, 3 mM MgSO4, 1 mM NaTri5VO4, 1 mM EGTA, 10 mM MOPS-Tris, pH 7.2, in the presence of 10 mM \[^{3H}\]JATP (Amersham Corp., specific activity: 36 Ci/mmol), varying concentrations of cold ATP, and 1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. After standing at 4°C for 20 min, bound and unbound ATP were separated by centrifugation in an Eppendorf centrifuge at 3,000 rpm for 5 min at 4°C. Samples were washed once in ice-cold binding buffer containing the amount of bound \[^{3H}\]JATP was determined by scintillation counting.

Equilibrium ATP Binding—Aliquots of 100–200 μg of SDS-treated gradient membranes were incubated at 0–4°C in 1 ml containing either 10 mM NaCl or 10 mM KCl, 10 mM MOPS-Tris, pH 7.2, 10 mM EDTA-Tris, and \[^{3H}\]JATP (Amersham Corp., specific activity: 36 Ci/mmol) and ATP-Tris to final concentrations of 0.2–300 mM. Bound and unbound \[^{3H}\]JATP were separated by centrifugation at 100,000 × g for 30 min at 4°C. The supernatant was discharged and remaining buffer removed with a paper towel. Bound \[^{3H}\]JATP was determined by scintillation counting, and specific binding was calculated as binding in presence of NaCl minus binding in presence of KCl.

ATPase Assay—For assay of Na,K-ATPase activity 10–20 μg aliquots containing 50–100 μg of protein were transferred to tubes containing 0.5 ml of 130 mM NaCl, 20 mM KCl, 3 mM MgCl2, 3 mM ATP, with or without 1 mM ouabain, 10 mM MOPS-Tris, pH 7.2, at 37°C. After incubation for 1, 3, 5, or 10 min at 37°C, the reaction was stopped with 1 ml of ice-cold 2% H2SO4, 0.1% ascorbic acid, 0.5% ammonium heptamolybdate, and 0.5% SDS. The tubes were left at room temperature for 15 min, and absorbance was measured at 690 nm as before (39). The Na,K-ATPase activity was calculated from the difference in absorbance for tubes with and without 1 mg ouabain.

Results

Development of a Host/Vector System for Na,K-ATPase Expression in S. cerevisiae—In the 2- γ-subunit plasmids constructed for expression of the pig kidney Na,K-ATPase, pPAP1466 and pPAP1666, the cDNAs encoding the \( \alpha_1 - \) and \( \beta_1 - \) subunits are transcribed from identical CYC-GAL promoters to avoid non-stochiometric transcription of the two subunit genes (Fig. 1). The CYC-GAL promoter is a strong galactose-inducible hybrid promoter containing the DNA sequences required for constitutive cytochrome \( c_1 \) (CYC1) expression and the upstream activating sequence from the GAL1-GAL10 intragenic promoter region (32). The \( \beta_2 \)-m origin of replication assures that the expression plasmid is stably maintained in the yeast cell. The expression plasmid carries two selective markers, URA3 and the poorly expressed allele of the LEU2 gene, leu2-d. A plasmid with two selective markers was favored for two reasons. First, manipulation of the plasmid copy number by altering the composition of the growth medium was facilitated, and second, homologous recombination between the identical CYC-GAL promoter regions transcribing the \( \alpha_1 - \) and \( \beta_1 - \) subunits, with loss of one of the subunit genes, was avoided. Selection for uracil autotrophy produces transformants with a plasmid copy number of around 20, while selection for both leucine and uracil autotrophy selects for growth of yeast cells with an extraordinary high copy number of around 200 (41).

The two expression plasmids differ in their E. coli origin of replication in that pPAP1466 is a pUC derivative (31) with a very high copy number, while pPAP1666 is a P15A-derived plasmid (34) with a 10-fold reduced copy number in E. coli compared with pPAP1466. The use of pPAP1666 with a low copy number in E. coli was necessary, as we were unable to clone various \( \alpha_1 - \) subunit mutations into pPAP1666.

The yeast strain BJ 5457 (20), lacking the PEP4 and PRB1 protease activities, was chosen as the basic host strain for the production of recombinant Na,K-ATPase in order to reduce the possibility of proteolytic degradation of the enzyme during synthesis and purification. The transactivating GAL4 protein is known to be limiting for expression controlled by galactose-regulated promoters (17). The GAL4 protein level can be increased by integrating a GAL10-GAL4 transcriptional fusion into the yeast chromosome (17). Therefore, we constructed the yeast-integrating plasmid pPAP1500. Addition of galactose to PAP1500 transformed with pPAP1466 or pPAP1666 should initiate a cascade reaction leading to expression of GAL4 protein and Na,K-ATPase \( \alpha_1 - \) and \( \beta_1 - \) subunits.

\[ \text{Equilibrium } [\text{H}]\text{Ouabain Binding to Intact Yeast Cells—Aliquots containing } 10 \text{ mg of intact yeast cells were incubated at } 37 \text{°C for } 1 \text{ h in } 1 \text{ ml sorbitol, } 3 \text{ mM MgSO}_4, 1 \text{ mM NaTri5VO}_4, 1 \text{ mM EGTA, and } 10 \text{ mM MOPS-Tris, pH } 7.2, \text{ in the presence of } 10 \text{ mM } [\text{H}]\text{Ouabain (Amersham Corp., specific activity: } 36 \text{ Ci/mmol)} \text{ and varying concentrations of cold ouabain. After standing at } 4 \text{°C for } 20 \text{ min, bound and unbound ouabain were separated by centrifugation in an Eppendorf centrifuge at } 3,000 \text{ rpm for } 5 \text{ min at } 4 \text{°C. Samples were washed once in ice-cold binding buffer. The amount of bound } [\text{H}]\text{Ouabain was determined by scintillation counting.} \]
Growth Physiology and Kinetics of Na,K-ATPase Expression in Yeast—The physiological effect of expression of the Na,K-ATPase on growth of yeast cells was determined in a series of growth experiments with PAP1500(pPAP1466). Fig. 3 illustrates how the yeast growth phase was separated from the Na,K-ATPase expression phase. In the growth phase, the level of Na,K-ATPase expression is low due to the absence of galactose. Later in the growth phase, the cells were grown with lactate as carbon source to avoid repression by glucose of the CYC-GAL promoters during the galactose-induced phase of Na,K-ATPase synthesis. In Fig. 3 the cells were grown under inducing and noninducing conditions in media selecting for a high plasmid copy number. Growth of the transformed yeast cells ceased immediately after induction of Na,K-pump biosynthesis with galactose whether the cells were expressing wild type or the D369N and D369A mutations.

Integration of a GAL10-GAL4 transcriptional fusion in the yeast chromosome had a dramatic effect on the level of expression of Na,K-ATPase, in media selecting for the high plasmid copy number. Fig. 4 shows that induction of Na,K-ATPase was much faster and that the maximal number of \([H]\)ouabain binding sites was about 10-fold higher in cells with the chromosomal fusion than in the yeast strain, with only a few copies of GAL4 protein/cell.

A number of experiments were performed to determine the influence of composition of the growth medium and induction time on the time course of appearance of high affinity \([H]\)ouabain sites measured in crude yeast membranes. Fig. 5 shows that the highest density of \([H]\)ouabain sites is seen in yeast cells, with a high copy number of the expression plasmid, and growing in synthetic minimal medium supplemented with all amino acids except leucine, tryptophan, and histidine. In medium without the supplement of amino acids, accumulation of Na,K-ATPase ceased after 48 h, and then the level decreased, suggesting that low levels of amino acids may be rate-limiting for synthesis of Na,K-ATPase.

Purification and Characterization of Na,K-ATPase from Crude Yeast Cell Membranes—Under optimum growth conditions in the computer-controlled bioreactor, the yield of yeast was 150–200 g/10 liters of culture, and the concentration of \([H]\)ouabain sites was 323 ± 31 pmol/g cells (mean ± S.E., n = 5 preparations). This value corresponds to 32,500 ± 3,000 sites/cell. The density of sites was the same in the intact transplanted yeast cells as in spheroplasts after removal of the cell wall by lyticase (data not shown).

In crude yeast membranes, the binding capacity for \([H]\)ouabain was 10–15 pmol/mg of protein. For characterization of the recombinant enzyme, it was essential to reduce the endogenous H-ATPase activity and to purify the enzyme by removal of extraneous protein. Fig. 6 shows the SDS curve required for application of the purification scheme developed previously for purification of the Na,K-ATPase from kidney (38, 39). In contrast to the 3–5-fold activation of Na,K-ATPase in kidney membranes after incubation with SDS, due to demasking of closed right-side-out vesicles, the activity of Na,K-ATPase or H-ATPase in yeast cell membranes increased only about 20%. At the membrane protein concentration of 2 mg/ml, an optimum concentration range for SDS was found (0.5–0.7 mg/ml) where most of the H-ATPase of the yeast cell membranes was inactivated, while \([H]\)ouabain binding and Na,K-ATPase activity of the recombinant enzyme were preserved. The peak of \([H]\)ouabain binding in crude yeast cell membranes was found at equilibrium densities in the range 1.15–1.2 g/ml.
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Fig. 5. Effect of composition of growth medium on accumulation of ouabain sites. Crude yeast membranes were isolated from strain PAP1500(pPAP1466) after induction with 2% galactose at time 0. Procedures for membrane preparation and [3H]ouabain binding were as in Fig. 4. Cells were grown with 0.5% glucose and 2% lactate as carbon source prior to induction, as illustrated in Fig. 3. Abbreviations used: -leu - aa, growth in the absence of leucine and the presence of lysine; + leu + aa, growth in the presence of leucine and lysine; -leu + aa, growth medium supplemented with all amino acids except leucine, tryptophan, and histidine.

Fig. 6. Demasking and inactivation of H-ATPase (●) and [3H]-ouabain binding (○) of crude yeast cell membranes during incubation with SDS in presence of ATP. Membrane protein at 4 mg/ml was incubated at 20°C in 200 μl with SDS at the indicated concentrations in a medium containing 1 mM EDTA, 3 mM Na2ATP, 10 mM MES-Tris, pH 7.0. For assay of vanadate-sensitive H-ATPase activity, 10-μl portions were transferred to test tubes and analyzed as described under "Materials and Methods." For assay of [3H]ouabain binding, portions of 25 μl containing 100 μg of membrane protein were transferred to centrifuge tubes containing 1 ml of binding medium containing 10 mM [3H]ouabain without or with 1 mM unlabeled ouabain. Other components and separation of free and bound ligand were as described under "Materials and Methods."

SDS in a lower concentration, 0.5 mg/ml SDS in the presence of 3 mM ATP, or at 0.3 mg/ml SDS in the absence of ATP.

Fig. 7. Scatchard plots of [3H]ouabain binding to crude yeast cell membranes isolated from strain PAP1500(pPAP1466) (●) and partially purified recombinant Na,K-ATPase (○). Aliquots of the preparations containing 100–200 μg of protein were incubated with [3H]ouabain and unlabeled ouabain at final concentrations ranging from 10.8 to 161 nM. The data were fitted by nonlinear least squares regression (line). The K0, and binding capacities were 21 ± 2 nM and 10.0 ± 0.5 pmol/mg of protein for the crude membranes and 11 ± 2 nM and 44 ± 2 pmol/mg of protein for the purified Na,K-ATPase.

Expression of Recombinant Na,K-ATPases without Turnover—We constructed two mutants in the α1 subunit from SF9 cells in expression system. The D369N mutation exchanges the asparagine, while the D807N mutation exchanges an aspartic acid potentially involved in cation binding for an asparagine. As expected both D369N (7, 18) and D807N (19) were devoid of activity.
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**Fig. 8.** Western blot of SDS-extracted membranes from yeast cells. Recombinant wild type αβ-units (lanes a-e), recombinant D369N αβ-units (lanes f-m), recombinant D807N αβ-units (lanes n-u) partially purified pig kidney Na,K-ATPase (lanes i, n-q, and v-z) after separation by SDS-polyacrylamide gel electrophoresis. The amount of pig kidney Na,K-ATPase is calculated as μg αβ-units assuming one αβ-unit per [3H]ouabain binding site (Ref. 35) as determined from Scatchard analysis, see “Materials and Methods.” Protein in lanes a, j, and r, 0.48 μg; in lanes b, k, and s, 1.0 μg; in lanes c, l, and t, 5.0 μg; in lanes d, n, and u, 10.0 μg; and in lane e, 20.0 μg. Na,K-ATPase αβ-unit in lanes f, n, and v, 0.016 μg; in lanes g, o, and x 0.031 μg; in lanes h, p, and y, 0.078 μg; and in lanes i, q, and z, 0.156 μg. Arrows indicate the position of the α-subunit. From this standardization, the purity in terms of α-subunit protein was estimated from scans of the blots to be 0.71% for wild type, 0.68% for D369N, and 0.54% for D807N.

**Fig. 9.** Na,K-ATPase activity of partially purified recombinant Na,K-ATPase as a function of Na+ and K+ concentrations. The reaction mixture contained 3 mM ATP at pH 7.5 and 37°C. Isotonicity was maintained by exchanging NaCl and KCl in the medium for assay of Na,K-ATPase described under “Materials and Methods.”

Na,K-ATPase and K-p-nitrophenyl phosphatase activity, and D369N was not phosphorylated from ATP (data not shown). Quantitative Western blotting (Fig. 8) showed that both mutations are expressed with the same concentration of α-subunit protein in the membrane as the wild type enzyme. Comparison with the purity estimated from [3H]ouabain binding also shows that the stoichiometry is one ouabain site/α-subunit for the two mutants. Data in Fig. 11 show that D369N and D807N had the same maximum binding capacity for [3H]ouabain as the wild type enzyme. The apparent affinity for ouabain of D807N (K_D = 13 ± 2 nM) was higher than for wild type Na,K-ATPase (K_D = 21 ± 2 nM), while that of D369N (K_D = 273 ± 13 nM) was much reduced. When the binding assay was conducted in the absence of vanadate, the mutant D369N and the wild type had dissociation constants in the same range (not shown) as an indication that D369N did not interact with vanadate. This agrees with the previous observation that D369N does not react with inorganic phosphate (7).

**Fig. 10.** Size-exclusion chromatography of recombinant Na,K-ATPase. The method was as described in Ref. 40 using 7.5 × 300-mm plus 7.5 × 75-mm TSK-gel G 3000 SW column equilibrated with C12E8, 5 mg/ml, in 150 mM potassium acetate, pH 6.0, and operated at 0.2 ml/min at 4°C. Prior to solubilization in C12E8 (Ref. 40), Na,K-ATPase was incubated with 100 nm [3H]ouabain, 3 mM MgSO_4_, and 1 mM NaTris_2VO_4_ as in Ref. 39.

Effects of Mutations to Asp369 on the Affinity for [3H]ATP Binding—The high yield of the preparation of recombinant Na,K-ATPase from yeast allowed assays not previously achieved for recombinant Na,K-ATPase, such as [3H]ATP binding at equilibrium (cf. Ref. 44) with the possibility of examining the effect of mutations on the binding constant of ATP to the E2 conformation. In agreement with the known properties of renal Na,K-ATPase (2, 44), addition of NaCl up to 10 mM did not alter binding of [3H]ATP to wild type or D369N in MOPS-Tris buffer, since both the unliganded and the Na+-bound E2 form of Na,K-ATPase have high affinities for ATP (K_a 0.1–0.2 μM). In contrast titration with KCl strongly reduced the level of binding of [3H]ATP at 10 mM with K_a 0.13 mM KCl for wild type and 0.2...
Expression of Na,K-ATPase in Yeast

Procedures for preparation of gradient membranes, incubation with SDS, and assay of $[3\text{H}]\text{ATP}$ in NaCl or KCl medium were as described under "Materials and Methods." The data were fitted by nonlinear least squares regression to the lines with $K_0 = 10^9 \pm 11 \text{ mM}$ and a binding capacity of $14.1 \pm 0.8 \text{ pmol/mg of protein}$ for wild type (WT) and $K_0 = 5.9 \pm 0.4 \text{ nm}$ and a binding capacity of $21 \pm 1 \text{ pmol/mg of protein}$ for D369N.

Fig. 12. Scatchard plot of $[3\text{H}]\text{ATP}$ binding to SDS-treated gradient membranes of wild type and mutant D369N recombinant Na,K-ATPase. Procedures for preparation of gradient membranes, incubation with SDS, and assay of $[3\text{H}]\text{ATP}$ in NaCl or KCl medium were as described under "Materials and Methods." The data were fitted by nonlinear least squares regression to the lines with $K_0 = 10^9 \pm 11 \text{ mM}$ and a binding capacity of $14.1 \pm 0.8 \text{ pmol/mg of protein}$ for wild type (WT) and $K_0 = 5.9 \pm 0.4 \text{ nm}$ and a binding capacity of $21 \pm 1 \text{ pmol/mg of protein}$ for D369N.

The combined effect of engineering these parameters was to increase the density of Na,K-pumps to $32,500 \pm 3,000$ sites/cell or $54 \pm 5 \mu\text{g}$ of Na,K-pump protein/g of yeast cell. In the crude membrane fraction from the yeast (10–15 pmol/mg of protein) and in the SDS-extracted membranes (42–50 pmol/mg of protein), the pump density was higher than achieved previously in eucaryotic cells, cf. Table I. The recombinant enzyme is fully active, with one site for binding of ATP and ouabain per $\alpha$-subunit and a range of molecular activities close to those of the native Na,K-ATPase of pig kidney. The relatively high activity and the lack of endogenous background allowed for analyses not previously achieved for recombinant Na,K-ATPase, such as ATP binding at equilibrium.

An important reason for separating the growth phase from the phase of Na,K-ATPase biosynthesis is the apparent toxicity of Na,K-ATPase protein synthesis. The evidence for this is the immediate arrest of cell growth and division following the galactose induction of Na,K-ATPase biosynthesis. This could be due to the activity of the Na,K-pump, but arrest of cell growth was independent of the enzymatic activity of the expressed pump as yeast strains expressing wild type or inactive mutants behaved identically. Also, the expression levels obtained in this work do not, in general, prohibit yeast cell growth. Much higher levels of constitutive heterologous expression has been described for soluble proteins (47). Also the endogenous GAL1 protein accumulates to 0.8% of total cell protein after galactose induction without affecting cell viability (48). It is therefore reasonable to propose that the toxicity of Na,K-ATPase protein synthesis is due to perturbation of the yeast membranes following the insertion of protein with hydrophobic membrane-embedded sequences. Previous experiments with membrane proteins, OmpA (49) and $\alpha$-hemagglutinin (50), demonstrated selection against the production of hydrophobic intramembrane segments in response to the poor tolerance by the host cell. Selection for low expressing variants has been observed in several cases, where constitutive promoters were used to drive expression of membrane proteins (51, 52). In the present expression system, the selection pressure was avoided through the use of a strong inducible promoter, but the toxicity of the expression suggests that the capacity of the yeast membrane system may set the limit for the concentration of Na,K-ATPase that can be achieved in these cells.
described for isofoms of the Arabidopsis thaliana H-ATPase using a multicopy plasmid and the strong constitutive yeast PMA-1 (plasma membrane H-ATPase) promoter (33). This expression system directs the plant proton pump to the endoplasmic reticulum membranes, where it constitutes 45% of the endoplasmic reticulum proteins, but the plant proton pump may also complement the proton pump in the yeast plasma membrane (53), like the Neurospora H-ATPase (54). In our experience, insertion of αβ-unit cDNA in this plasmid did not lead to expression of significant amounts of Na,K-ATPase. The constitutive PMA-1 host/vector system (33) also proved inefficient for expression of the SR-Ca-ATPase (55). The Na,K-ATPase expression system in yeast described by Horowitz et al. (1) also used constitutive promoters and achieved only low expression levels, 0.05% of the plasma membrane protein, cf. Table I. It seems that the high expression level obtained by constitutive expression of the plant H-ATPase is the exception, while a high expression level for Na,K-ATPase and SR-Ca-ATPase is incompatible with constitutive promoters. A probable explanation could be that insertion of H-ATPase into yeast membranes is less prone to eliciting selection for low expressing variants than is expression of the Na,K- or Ca-ATPases. In agreement with these notions, the SR-Ca-ATPase has recently been expressed in a relatively high concentration, 0.3% in the yeast membranes, under control of a galactose-regulated promoter (55).

For the purification of the recombinant Na,K-ATPase, it was important to establish conditions where the plasma membrane H-ATPase could be removed. This enzyme constitutes background activity in the enzyme assay and [3H]ATP binding experiments. Fortunately, the H-ATPase turned out to have a relatively high sensitivity to denaturation by incubation with SDS. Our data show that Na and K dependence of ATP hydrolysis, and ATP binding, of the recombinant enzyme are similar to the kinetics of the native renal Na,K-ATPase. In the best of our preparations, the molar activity was close to 7,000 P/min, but the number varied in the range 4,500–7,000 P/min for reasons that are not yet fully understood. A variation in molecular activity is also apparent among the previous preparations from yeast, in the range from 792 to 8212 P/min (1, 15, 56, 57), cf. Table I. The relatively large scatter of the reported molecular activity among preparations from different host systems may reflect difficulties in determining site concentrations in preparations with relatively low activity. The highest expression in terms of α-subunit protein is achieved in baculovirus-infected SF-9 cells (9), but only about 3% of the protein is active in terms of Na,K-ATPase activity and ligand binding, Table I. The insect cells (9, 43), as well as the COS-1 (5) and HeLa (10, 20, 58) cells, also express endogenous Na,K-ATPase activity as denoted in Table I.

The yeast expression system clearly does not distinguish active Na,K-ATPase from inactive mutant αβ-units with respect to biosynthesis and translocation to the cell membranes. The D369N and D807N mutations were expressed in yeast membranes at concentrations of α-subunit and [3H]ouabain binding sites that are comparable with those of the wild type enzyme. For the D369N mutant it was further demonstrated that the concentration of protein expressed in the yeast membranes was equal to the concentration of [3H]ATP sites. The αβ-units of the D369N mutant and recombinant Na,K-ATPase have the same hydrodynamic properties as purified Na,K-ATPase from kidney.

Mutation at the phosphorylation site of the negatively charged aspartic acid 369 for an asparagine residue caused a remarkable decrease in dissociation constant of the protein-ATP complex. This is most likely due to a true change in the binding constant of the E1NaK conformation for ATP and not to a shift in conformational equilibrium. Our ATP binding data for D369N demonstrate that Na+ and K+ stabilize the E1Na and E1K conformations with widely different affinities to ATP. In addition, the D369N mutant has the same affinity for [3H]ouabain as the wild type in the presence of Mg2+. Previous data also show that the [3H]ouabain complex of the mutant responds to Na+ and K+ in the same manner as the wild type Na,K-ATPase (7). In the Ca-ATPase from SR, modification of either Asp351 or the neighboring Lys352 abolishes phosphorylation (59). Our data show that reduction of the negative charge of Asp369 greatly increases the affinity for ATP. Although the negatively charged Asp residue is essential for enzymatic turn-over, it may thus reduce the affinity for ATP through electrostatic repulsion of the negatively charged γ-phosphate present on the ATP molecule.

The extraordinary high affinities of D369N or D369A for ATP turn these mutants into powerful tools for future identification of residues involved in ATP binding to Na,K-ATPase and for studying the influence of Mg2+ upon ATP binding. Given the high degree of homology of the amino acid sequence around the phosphorylation site (ICS D KTGTLT), it can be expected that the large increase in affinity for ATP upon reducing the negative charge on the phosphorylated side chain will be a general phenomenon for the P-type cation pumps, H,K-ATPase, Ca-ATPase, and H-ATPase.

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Table I

| Ref. | Host cell | Na,K-ATPase activity | Ligand binding capacity | Molecular activity |
|------|-----------|----------------------|------------------------|--------------------|
|      |           | Transfected | Endogenous | [3H]Ouabain | Phosphorylation | Pi·min\(^{-1}\) |
| Present data | Yeast | 307 | 0 | 43 | 7139 |
| 1, 15, 56 | Yeast | 15–44 | 0 | 1–5 | 792–1445 |
| 57 | Yeast | 8–24 | 0 | 1–3 | 8212 |
| 9, 43 | SF-9 | 88 | 2 | (2)\(^a\) | 12.5 | 6723 |
| 5 | COS-1 | 148–543 | 378 | | |
| 10, 20, 58 | HeLa | 125–283 | 148 | | |
| 7 | NIH 3T3 | 57 | n.d. | | |
| 14 | Xenopus | 73 | 22 | | |

\(^a\) [3H]Ouabain binding determined for α2 and α3 isoforms.

\(^b\) Calculated from specific activity and molecular activity.
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