Expression of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in Pichia pastoris

ANALYSIS OF WILD TYPE AND D369N MUTANT PROTEINS BY Fe\textsuperscript{2+}-CATALYZED OXIDATIVE CLEAVAGE AND MOLECULAR MODELING

Received for publication, July 30, 2003, and in revised form, August 27, 2003

Published, JBC Papers in Press, August 29, 2003, DOI 10.1074/jbc.M308303200

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Na\textsuperscript{+},K\textsuperscript{+}-ATPase (pig α1,β1) has been expressed in the methylotrophic yeast Pichia pastoris. A protease-deficient strain was used, recombinant clones were screened for multicopy genomic integrants, and protein expression, and time and temperature of methanol induction were optimized. A 3-liter culture provides 300–500 mg of membrane protein with ouabain binding capacity of 30–50 pmol mg\textsuperscript{-1}. Turnover numbers of recombinant and renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase are similar, as are specific chemotryptic cleavages. Wild type (WT) and a D369N mutant have been analyzed by Fe\textsuperscript{2+} and ATP-Fe\textsuperscript{2+}-catalyzed oxidative cleavage, described for renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Cleavage of the D369N mutant provides strong evidence for two Fe\textsuperscript{2+} sites: site 1 composed of residues in P and A cytoplasmic domains, and site 2 near trans-membrane segments M3/M1. The D369N mutation suppresses cleavages at site 1, which appears to be a normal Mg\textsuperscript{2+} site in E\textsubscript{2} conformations. The results suggest a possible role of the charge of Asp\textsuperscript{369} on the E1 \rightarrow E\textsubscript{2} conformational transition. A molecular dynamic simulation of ATP-Mg\textsuperscript{2+} binding to WT and D351N structures of Ca\textsuperscript{2+}-ATPase (analogous to Asp\textsuperscript{369} of Na\textsuperscript{+},K\textsuperscript{+}-ATPase) supplies possible explanations for the new cleavage and for a high ATP affinity, which was observed previously for the mutant. The Asn\textsuperscript{351} structure with bound ATP-Mg\textsuperscript{2+} may resemble the transition state of the WT poised for phosphorylation.

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase utilizes the free energy of hydrolysis of ATP to actively transport three intracellular Na\textsuperscript{+} ions and two extracellular K\textsuperscript{+} ions in opposite directions across animal cell membranes. The Na\textsuperscript{+},K\textsuperscript{+}-ATPase is a member of the P-type family of cation pumps. The kinetic mechanism of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, as of other P-type pumps, involves a phosphoenzyme intermediate and is now largely understood (1, 2). As pointed out by Jencks (3), strict cation and substrate specificities of the phosphorylation and dephosphorylation reactions, and tight coupling of the E\textsubscript{1} \rightarrow E\textsubscript{2} conformational changes to cation movements are the essential features of all P-type ion pump mechanisms. These central questions of the energy transduction mechanism of P-type pumps can now be posed in structural terms (4) because of availability of molecular structures of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase for both E\textsubscript{1},2Ca\textsuperscript{2+} and E\textsubscript{2} conformations (5, 6).

The Ca\textsuperscript{2+}-ATPase molecule consists of head, stalk, and membrane sectors (5). There are 10 transmembrane segments in the membrane domain with two Ca\textsuperscript{2+} ions ligated approximately in the center of the bilayer and between transmembrane segments M4, M5, M6, and M8 in the E\textsubscript{1},2Ca\textsuperscript{2+} conformation. The stalk sector consists of three domains, nucleotide binding (N),\textsuperscript{1} phosphorylating (P), and anchor or actuator domain (A). Comparison of the crystal structure an E\textsubscript{1},2Ca\textsuperscript{2+} and E\textsubscript{2} conformations shows that in the E\textsubscript{2} conformations the N, P, and A domains are separate, whereas in E\textsubscript{1} conformations the domains are gathered together, moving essentially as rigid bodies (5, 6). Movement of the A domain toward P and N domains in the E\textsubscript{1} \rightarrow E\textsubscript{2} transition is associated with a bending of S5 that entails complex movements of several transmembrane segments. This changes the ligation of the occluded Ca\textsuperscript{2+} ions within the transmembrane segments allowing them to dissociate within the sarcoplasmic reticulum. Whereas this general paradigm clearly applies to the other P-type pumps, not all features are explained by the crystal structures. As one example, phosphorylation by ATP requires close proximity of the nucleotide binding N and phosphorylation P domains, but this is not observed in the E\textsubscript{1},2Ca\textsuperscript{2+} (Protein Data Bank code 1EUL) structure. In addition, of course, there are the specific features of other ion pumps particularly the cation selectivities, β subunits in the case of Na\textsuperscript{+},K\textsuperscript{+}-ATPase or H\textsuperscript{+},K\textsuperscript{+}-ATPase (7), or FXYD proteins, which are accessory subunits of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (8).

For analysis of conformational transitions and ligand binding on renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase or gastric H\textsuperscript{+},K\textsuperscript{+}-ATPase specific oxidative cleavage catalyzed by bound Fe\textsuperscript{2+} or the ATP-Fe\textsuperscript{2+} complex has turned out to be very informative (see Refs. 9–13 and a recent review in Ref. 14). Recently this technique has also been applied to Ca\textsuperscript{2+}-ATPase (15) and H\textsuperscript{+},K\textsuperscript{+}-ATPase (16). The cleavages provide information on the spatial organization surrounding the bound Fe\textsuperscript{2+}, or ATP-Fe\textsuperscript{2+} (14). One major prediction from the cleavages catalyzed by bound Fe\textsuperscript{2+} ions is that E\textsubscript{1} \leftrightarrow E\textsubscript{2} conformational changes are associated with large movements in the cytoplasm domains, N, P, and A domains.

\textsuperscript{1} The abbreviations used are: N, nucleotide binding domain; P, phosphorylating domain; A, anchor or actuator domain; A0X1, alcohol oxidase 1; AMP-PNP, 5′-adenosyl-β,γ-imidodiphosphate; DTPA, diethyl enetriaminedipentacetic acid 1-amido-fluoresceinamine, isomer I; PMSF, phenylmethylsulfonyl fluoride; Desferal, desferrioxamine mey late; YNB, yeast nitrogen base; WT, wild type; BMW, buffered minimal glycerol; BMM, buffered minimal methanol medium; MOPS, 4-morpholinonepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.

This paper is available on line at http://www.jbc.org
interacting in $E_2$ states but separate in the $E_1$ state (14). This is fully consistent with the difference observed between the crystal structure of Ca$^{2+}$-ATPase in the $E_2$2Ca$^{2+}$ (Protein Data Bank code 1EUL) or an $E_2$ complex (Protein Data Bank code 1KJU) (Refs. 5 and 17, see also Ref. 14) for a discussion of differences between the 1KJU structure and the new $E_2$ structure I1WO). The Fe$^{2+}$ in ATP-Fe$^{2+}$ substitutes for Mg$^{2+}$ ions in catalyzing phosphorylation and ATPase activity (18, 19). Thus cleavages mediated by ATP-Fe$^{2+}$ provide information on ligation of Fe$^{2+}$ or Mg$^{2+}$ ions as well as N, P, and A domain proximity. The high selectivity of the ATP-Fe$^{2+}$-mediated cleavages, as well as recent observations of similar cleavages mediated by a fluorescein-DTPA-Fe$^{2+}$ complex (13), suggest that cleavages occur only next to residues that bind the Fe$^{2+}$ directly. Thus, overall, the cleavages have led to a picture of N, P, and A domain interactions and ATP-Mg$^{2+}$ or Mg$^{2+}$ ions binding in the different conformational states of the catalytic cycle (summarized in Refs. 4, 13, and 14). In another recent development the masses of cleavage fragments have been measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (14, 20). With the exception of two fragments with exactly known N termini (213ESE and 713VND), other fragments could not be sequenced, and the N termini were designated previously as near XXXX by comparison with known proteolytic fragments. The analysis by mass spectrometry has now greatly improved the assignments, so that the designation of all N termini of fragments as near XXXX is accurate to within 2

Bowman et al. (11) showed that Asp710 is a Mg$^{2+}$ binding residue (21).

Unfortunately, the Ca$^{2+}$-ATPase structures and modeling based on the structures are invaluable for testing the validity of interpretations, cleavage experiments also lead to inferences on ATP and Mg$^{2+}$ binding and N to P domain interactions not available from the crystal structures. Thus, it is highly desirable to investigate the predicted roles of specific residues by mutational analysis. As one relevant example, mutations of residues in the conserved 707TGDVNDS sequence in the P domain have already shown that Asp$^{710}$ is a Mg$^{2+}$ binding residue (21).

We describe here expression of Na$^{+}$,K$^{+}$-ATPase in the methylophylic yeast Pichia pastoris and analysis of the wild type protein and the D369N mutant by Fe$^{2+}$-catalyzed oxidative cleavage. The a1 and b1 subunit of cDNA, under control of the AOX1 promoter, are incorporated into the alcohol oxidase chromosomal locus (AOX1), and protein synthesis is induced with methanol (see Ref. 22 for a general review). P. pastoris can grow to high cell densities and produce high levels of secreted soluble proteins. As a precedent for expression of an integral membrane protein in P. pastoris, the P-glycoprotein (MDR3) has been expressed in large quantities and purified for biochemical and structural work (23, 24). Thus, expression of functional Na$^{+}$,K$^{+}$-ATPase in P. pastoris at significant levels appeared to be an attractive possibility. Na$^{+}$,K$^{+}$-ATPase has been expressed in many cell types, and used extensively for structure-function analysis, but of most relevance to the present work wild type and mutant Na$^{+}$,K$^{+}$-ATPase proteins have been expressed in Saccharomyces cerevisiae and interactions of the pump ligands, ATP, Na$^{+}$, K$^{+}$, Mg$^{2+}$, and ouabain characterized in detail, particularly by direct binding assays (21, 25–29). On the other hand, analysis of structural organization and conformational changes by proteolytic cleavage or metal-catalyzed oxidative cleavage has not been described.

As a first step in analysis of mutants by oxidative cleavage we have chosen to examine the active site mutant, D369N. Previously, the D369N and D369A mutants of pig a1b1 Na$^{+}$,K$^{+}$-ATPase complex were expressed in S. cerevisiae and analyzed in detail at the biochemical level (26). The mutant proteins are, of course, inactive because of the inability to undergo phosphorylation, but they show two striking properties. First, the ATP binding affinity of the D369N and D369A mutants is much higher than that of the WT protein. A similar effect of D351N, A and T mutants of Ca$^{2+}$-ATPase has also been described (20). Second, the conformational equilibrium $E_2 \rightarrow E_1$ is significantly poised toward the $E_2$ forms compared with the WT. However, the structural consequences of the mutations have not been investigated. In view of the central role of the active site aspartate, evidence on changes in structural organization caused by the D369N mutation could provide important information on the energy transduction mechanism.

**Experimental Procedures**

**Materials**

Escherichia coli HB101 (hids20 [rpsL - mcr], recA13, ara-14, proA2, lacY1, galR2, rplA20 (Smr$^\text{r}$), xyl-5, intI1, supE44, λ $^\text{if}$) was used for propagation and preparation of various plasmid constructs. P. pastoris strains GS115 (his4) and SMD1165 (his4, prb1) were used for transformation with the expression vector pHIL-D2(a/b). YNB medium (without amino acids, with ammonium sulfate) was obtained from Difco. $^{[3]}$H]Ouabain (TRK429) and $[^{32}$P]ATP (PB 10132) were obtained from Amersham Biosciences. α-Chymotrypsin (catalog number 2307) was obtained from Merck. All other chemicals were of analytical grade.

**Construction of Expression Vector, pHIL-D2(a/b)**

cDNAs encoding porcine a1 (accession number X03938) in plasmid pGEM and porcine b1 (accession number X04635) in plasmid pHIL2 were provided by P. L. Jorgensen (Copenhagen University). Plasmid pGEM1(a1) was digested with XbaI, HindIII, and DraI, and treated by Klenow. The XbaI-HindIII fragment containing a1 was then ligated with the large fragment released from the E. coli P. pastoris shuttle vector, pHIL-D2 after EcoRI, Klenow, and alkaline phosphatase treatment. The right orientation of the cloned fragment under the AOX1 promoter was determined by restriction analysis. Next, the β1 open reading frame and flanking regions (in plasmid pHIL2) were amplified by PCR using two synthetic oligonucleotides: sense, 5′-cccgaatttctgtgcgccggccgccacatccgggct and antisense 5′-atatagctttgtctgtctatctacctagatcaag containing EcoRI sites (underlines). The PCR fragment was digested by EcoRI and ligated to EcoRI-treated plasmid pHIL-D2 to produce pHIL-D2β1. A large part of the β1 gene was then replaced (using SacII and SnaBI) by the original gene, and the remaining PCR-generated region was sequenced. To construct a vector encoding both genes (a1 and b1), an additional selection marker has been introduced; the kan$^\text{a}$ gene that confers resistance to kanamycin in bacteria and to G418 in yeast cells.
The bacterial plasmid, pCV3 (kan<sup>R</sup>) prepared in E. coli SCS110<sup>Δmdm</sup>- (Stratagene) was digested with StuI and a fragment containing kan<sup>R</sup> was ligated to the SnaBl-digested plasmid pHIL-D2β1 to produce plasmid pHIL-D2β1( kan<sup>R</sup>). Next, the P<sub>Δylkl</sub>β1 kan<sup>R</sup> fragment was excised from pHIL-D2β1( kan<sup>R</sup>) by NotI and ClaI (partial digestion). The appropriate NotI-ClaI fragment (3,670 bp) was treated by Klencow and ligated to plasmid pHIL-D2α1 that was partially digested with Nael and dephosphorylated to produce the final expression vector pHIL-D2αβ1( 15,322 bp). This plasmid was used to mutate Aasp<sup>345</sup> in the α1 gene (D868N) by oligonucleotide-directed, site-specific mutagenesis using the overlap-extension PCR method (31). The PCR product was sequenced.

### Yeast Transformation

10 μg of linear DNA obtained by digestion of plasmid pHIL-D2αβ1 with NotI was used to transform spheroplasts of <i>P. pastoris</i> GS115 or SMD1165, according to the Invitrogen manual (version F). Approximately 120–150 His<sup>+</sup> prototrophs were plated to MM (1.34% YNB, 4 × 10<sup>-6</sup>% biotin, 0.5% methanol, 2% agar) and MD (1.34% YNB, 4 × 10<sup>-6</sup>% biotin, 2% dextrose, 2% agar) plates for Mut<sup>+</sup> screening (methanol selection). Transformation efficiency was assessed by plating 0.1% of the bead beater was filled with 50% glycerol at 20 °C. Unbroken cells and heavy membranes were removed by centrifugation at 10,000 × g for 10 min and light membranes were collected at 100,000 × g for 1 h. The pellet was suspended in 10 μM MOPS/Tris-HCl, pH 7.2, 1 mM EDTA, 25% glycerol with protease inhibitors (as above), and stored at −80 °C. Renal Na<sup>+</sup>–K<sup>+</sup>-ATPase was prepared as described in Ref. 33.

### Membrane Preparations

<i>P. pastoris</i> cells were collected by centrifugation and the pellet was resuspended in an ice-cold buffer containing 1.4 m sorbitol, 10 mM MOPS/Tris-HCl, pH 7.2, 1 mM EDTA, plus protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml chymotrypsin), at 1 g of cells/10 ml of buffer. For cell lysis, the cells were mixed with an equal volume of glass beads (0.5 mm) and disrupted by 8 × 1-min cycles using a Glass Bead Beater (Biospec Products, Inc.). The cooling jacket of the bead beater was filled with 50% glycerol at 20 °C.

### Growth and Media

<i>P. pastoris</i> GS115 and SMD1165 were grown according to the instructions in the Invitrogen Pichia Expression kit manual. Plating and screening transformants in RD medium (1 μm sorbitol, 2% dextrose, 1.34% YNB, 4 × 10<sup>-6</sup>% biotin, 0.005% of each amino acids: t-glutamic acid, t-methionine, t-lysine, t-leucine, and t-isoleucine), MD, and MM media were performed according to the manual. Liquid cultures were grown in BMG (buffered minimal glycerol medium, consisting of 1.34% YNB, 2% dextrose, 0.5% methanol, 2% agar) plates for Mut<sup>+</sup> colonies were grown overnight at 30 °C in 200 μl of YPD medium in 96-well plates. 5-μl Aliquots were then spotted onto a nitrocellulose filter (0.45 μm, Millipore) placed on the surface of a fresh YPD plate. After evaporation of the liquid, the plates were inverted and incubated at 30 °C for 24 h. Cell lysis and DNA fixation were done according to Ref. 32. A StuI-SacII fragment of the denaturated salmon sperm DNA was sequenced.

### DNA Dot Blot Screens

Mut<sup>+</sup> colonies were grown overnight at 30 °C in 200 μl ofYPD medium in 96-well plates. 5-μl Aliquots were then spotted onto a nitrocellulose filter (0.45 μm, Millipore) placed on the surface of a fresh YPD plate. After evaporation of the liquid, the plates were inverted and incubated at 30 °C for 24 h. Cell lysis and DNA fixation were done according to Ref. 32. A StuI-SacII fragment of the denaturated salmon sperm DNA was sequenced.

### SDSPAGE and Western Blots

12–25 μg of yeast membranes were mixed with 5-fold concentrated sample buffer containing 1 mM PMSF, and separated on 7.5 or 10% Tricine SDSPAGE (34). Proteins were blotted to a polyvinylidene difluoride membrane and immunostained with anti-Lys<sup>345<sup>Δylkl</sub>β1<sup>R</sup>-KETTY antibodies, 1:3,000 that recognize the C terminus of the α subunit, or with anti-β antibodies raised against the extracellular domain of the β subunit (35). Blots were visualized by enhanced chemiluminescence (ECL Plus kit) using anti-rabbit IgG horseradish peroxidase-conjugates as instructed (Amersham Biosciences). Immunoblots were scanned with an imaging densitometer (GS-890, Bio-Rad) and analyzed using the Multi-analyst software (Bio-Rad).

### Biochemical Assays

Ouabain binding to membranes or to whole cells was done using <sup>3</sup>Houabain essentially as described in Refs. 25 and 26. In assays of ouabain binding to membranes, at the end of incubation 3 ml of ice-cold 10 mM Tris-HCl was added, the samples were filtered on Whatman GF/C filters and washed twice with the same buffer. Na<sup>+</sup>–K<sup>+</sup>-ATPase activities were assayed using <sup>γ</sup>-<sup>32</sup>PATP as described in Ref. 33. Prior to both ouabain binding and ATPase assays, membranes (2 mg/ml) were incubated with 0.3 mg/ml SDS at room temperature for 30 min and then centrifuged. This procedure inactivates ouabain-inhibitable ATPase and demasks any Na<sup>+</sup>–K<sup>+</sup>-ATPase molecules in closed vesicles (36).

### Chymotryptic Cleavage (see Refs. 10 and 36)

Renal Na<sup>+</sup>–K<sup>+</sup>-ATPase or membranes containing the recombinant enzyme were centrifuged, and re-suspended in a medium containing 10 mM Tris-HCl, pH 7.4, and either 20 mM NaCl or 20 mM RbCl, at a concentration of 0.1 mg of protein/ml. The membranes were incubated for 10 min at 37 °C with 5 μg/ml α-chymotrypsin. The digestion was arrested by addition of 140 mM KCl plus 1 mM PMSF, and centrifugation. The pellets were re-suspended in 10 mM Tris-HCl, pH 7.4, and 1 mM PMSF. Cleavage fragments were visualized by immunoblotting using the anti-KETTY antibody.

### Oxidative Cleavage Reactions

Recombinant membranes were centrifuged and re-suspended (0.5 mg/ml) in media containing 20 mM MES (Tris), pH 6.5, with or without 130 mM NaCl or RbCl, or ATP, AMP-PNP, or other ligands as indicated in the figure legends. To 25 μl of the membrane suspension, freshly prepared solutions of 10 μM ascorbate, and different concentrations of FeSO<sub>4</sub> were added, then 10 mM H<sub>2</sub>O<sub>2</sub> was added to a total volume of 50 μl, and the suspension was incubated at 0 °C for 15 min. To arrest the reaction, 25 μl of a solution of EDTA, desferrioxamine mesylate (Desferal), and PMSF were added to final concentrations of 30, 3, and 1 mM, respectively. After 5 min at room temperature, 25 μl of 5× concentrated sample buffer were added and samples were loaded onto gels. Cleavage fragments were visualized by immunoblotting using the anti-KETTY antibody.

### Molecular Dynamics

The initial three-dimensional model of the wild type Ca<sup>2+</sup>–ATPase was truncated to comprise only the P and N domains, including bound ATP and Mg<sup>2+</sup>, as described in a recent publication (13). The D351N

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* A. Selivanov and E. Bibi, unpublished data.
mutation was performed using the SwissPdbViewer. The Asp351 side chain was manually adjusted to remove clashes and to point the side chain N-atom in the direction of the ATP. The simulations were performed with the GROMACS simulation package (37, 38), using the GROMOS96 united atoms force field (39) and the SPC water model (40). The truncated protein model was solvated with 14,764 molecules of water and a physiological ion concentration of Na$^+$ and Cl$^-$ ions in a cubic box with a minimum distance of 1.0 nm between protein and box boundaries. The excess negative charge of the protein model was neutralized by an excess of sodium ions, so that the system was electro-neutral. Periodic boundary conditions were applied. Long range electrostatic interactions were treated with PME approximation (41, 42). Van der Waals and short range electrostatic interactions were cut off at 0.9 nm. The system was coupled to an external water bath at 300 K and an external pressure reservoir set to 1 bar. The solvated system was minimized in 500 steps of steepest decent minimization, followed by a short 10-ps simulation of the system with position restraints on the protein and the ATP with a step size of 0.002 ps. This was followed by a 900-ps simulation of the whole system without restraints, again with a step size of 0.002 ps. The bonds of the system were restrained with the SHAKE algorithm (43).

RESULTS

Transformation of *P. pastoris* and Screening for Expression of α and β Subunits—The pig α1 and β1 cDNAs were cloned into the pHIL-D2 expression vector, each under control of the AOX1 promoter. The genes are incorporated into the endogenous AOX1 chromosomal locus by homologous recombination at the 5′ and 3′ ends. By placing the Hisα selectable marker between the α1 and β1 genes the possibility of recombination leading to insertion of the β1 gene without the α1 gene was avoided. Transformation of the wild type *P. pastoris* strain GS115 with the pHIL-D2 (αβ) expression vector led to appearance of degraded fragments of the α1 subunit. Therefore all experiments were done with a protease-deficient strain of *P. pastoris*, SMD1165, transformed with the pHIL-D2 (αβ) vector or the “empty” vector, pHIL-D2, as a control. Colonies were selected for histidine prototrophy, and then for the Mutα phenotype, which detects clones in which the AOX1 gene is disrupted by integration of the genes of interest, and as a result of which they grow slowly because of their dependence on the AOX2 gene for metabolism of methanol (22). Between 10 and 20% of the Hisα prototrophs showed the Mutα phenotype. In the experiment of Fig. 2, 11 such Mutα clones were grown for 5 days, either as colonies on plates, or in 5 ml of BMM liquid medium, with daily addition of 0.5% methanol. Fig. 2A shows results of the dot blot assay for the α1 DNA content, and Fig. 2B shows an immunoblot to detect the α1 subunit in the membranes prepared from these 11 clones. It is striking that the different clones showed a wide variation in content of α1 DNA and α1 subunit protein and that, qualitatively, there was good agreement between the DNA content and level of α1 subunit expressed. The empty vector control, of course, showed no expression of the α1 subunit. It is known that transformation of *P. pastoris* can result in multicopy integration into the AOX1 locus (32). The result in Fig. 2 is consistent with the assumption of multicopy integration, and shows that screening Mutα clones reveals those expressing the highest level of DNA or protein, which can then be used for optimization of expression (e.g. clone 91). As seen in Fig. 2B the α1 subunit expressed in the yeast membranes runs slightly behind the α1 subunit of renal Na$^+$,K$^+$-ATPase. This could indicate that post-translational clipping off of the first five residues MGKGV, which occurs in native renal cells, does not occur in the yeast cells. Fig. 2C shows that the β1 subunit is expressed in clone 91 as two quite sharp bands of apparent mass 47 and 44 kDa, respectively, by contrast with the heavily glycylated β subunit of the renal Na$^+$,K$^+$-ATPase. The finding in Fig. 2C demonstrates that the β1 subunit is only lightly glycosylated in the *P. pastoris*. Light glycosylation of heterologously expressed proteins is another known feature of *P. pastoris* (44).

Optimization of Expression of Na$^+$,K$^+$-ATPase—A series of experiments was carried out in flask cultures to determine the optimal stage of growth in the BMM prior to methanol induction, the time course after induction with methanol, the temperature in the methanol induction phase, and different growth media. Western blots, using the anti-KETY antibody, ouabain binding on crude membranes, or ouabain binding on whole cells, were used to assess the level of expression of the protein. Initial experiments, in which *P. pastoris* clone 91 (see Fig. 2) was grown to different densities in the BMM media, from the log to stationary phase, the glycerol was removed, and then methanol was added for 5 days, which showed optimal expression for log phase cultures (Fig. 2A–C). If glycerol was not removed prior to induction with methanol, expression was greatly suppressed. Thus, optimization of the time course of methanol induction utilized log phase cultures after removal or utilization of glycerol. Fig. 3 shows a time course of methanol-induced expression, for a 3-liter scaled-up culture in a Spinner flask at 25 °C. Maximal expression of the α1 subunit, and ouabain binding in crude membranes occurred after 6 days of methanol induction. Methanol induction in a basal salt (see Invitrogen *Pichia* Expression kit) medium did not lead to significant expression. Thus all experiments utilized the BMM growth medium and 6 days of induction. In experiments to examine the optimal temperature for the methanol induction phase, expression of the α1 subunit or ouabain binding in crude membranes was found to be similar at 30 and 25 °C, somewhat lower at 20 °C, while there was no expression at either 15 or 37 °C. A more detailed comparison of growth at 25 and 30 °C revealed an interesting phenomenon (Fig. 4). At 25 °C the cells grew to about twice the density compared with that at 30 °C and, although the ouabain binding to crude membranes was similar, ouabain binding to whole cells was about 3-fold higher for cells grown at 30 °C (see “Discussion”). For most purposes, cells were grown in the Spinner flasks at 25 °C to maximize the yield of crude membranes. Mechanical disruption of cells from 3 liters of culture, −100 g wet weight, provided about 500 mg of crude membranes with specific ouabain binding capacities of 30–50 pmol mg of protein$^{-1}$.  

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**Fig. 2.** Screening of Mutα clones for integration of cDNA and expression of α1 and β subunits. (A) Semi-quantitative DNA dot blot of 11 Mutα transformants. V, vector. (B) Western blot of membranes (25 µg) derived from 11 Mutα clones, probed with anti-KETYY, 1:3000. NKA, pig kidney Na$^+$,K$^+$-ATPase, 0.25 µg. (C) Western blot of β subunit expression in clone 91 and pig kidney Na$^+$,K$^+$-ATPase is shown.
activities at 37 °C compared at 25 and 30 °C. The measured range of Na+/H+ ratio of 1 corresponds to a Na+ to H+ ratio of 1 in a hemacytometer. Upper panel, Western blot using anti-K channel antibody from clone 91. Lower panel, ouabain binding to membranes done at 1 μM ouabain.

FIG. 3. Time course of methanol-induced expression of Na+,K+-ATPase. Three liters of culture were grown in a 3-liter Spinner flask. After methanol induction samples were removed every 24 h and membranes were prepared. Upper panel, Western blot using anti-K channel antibody from clone 91. Lower panel, ouabain binding to membranes done at 1 μM ouabain.

Turnover Number of the Recombinant Na+,K+-ATPase—Ouabain binding and Na+,K+-ATPase activity of the expressed protein has been determined and the turnover number calculated. Scatchard plots of ouabain binding to different crude membrane preparations gave dissociation constants of 17–21 nM and Bmax values in the range of 30–50 pmol mg of protein−1 (see also Table I). The measured range of Na+,K+-ATPase activities at 37 °C was 0.17–0.23 μmol mg of protein−1 min−1.

Using the ouabain binding Bmax values, as a measure of the site concentration, the range of calculated turnover numbers at 37 °C was 5666–7600 min−1. The higher values of turnover number are close to that of the renal Na+,K+-ATPase (33) and indicate that a large percentage of the recombinant protein is functional. Attempts to purify the recombinant enzyme by the SDS/ATP procedure of Jorgensen (33) were not successful, because of inactivation by the SDS. The ouabain binding capacity of the D369N mutant, which is discussed below, was 24 pmol mg of protein−1 and, of course, it has no ATPase activity.

Chymotryptic Cleavage of Recombinant Na+,K+-ATPase—The immunoblot in Fig. 5 shows that well characterized fragments generated by specific chymotryptic cleavage of renal Na+,K+-ATPase (36), in E1Na and E1K(O) conformations, are observed also for the recombinant protein. The yield of fragments is somewhat lower for the recombinant protein, a feature seen also for Fe2+-catalyzed cleavage, as discussed below. The experiment indicates that native structural organization of the renal enzyme is maintained in the recombinant Na+,K+-ATPase.

Fe2+-catalyzed Oxidative Cleavage of WT and D369N Mutant—Fig. 6 presents results of Fe2+-catalyzed oxidative cleavage at 0 °C of the wild type Na+,K+-ATPase, and the D369N mutant, both in E1Na- and E1K(O)-containing media, respectively (see Refs. 9 and 10). Equal quantities of WT and D369N mutant protein were applied to the gel. The fragments of the recombinant proteins are identified in immunoblots by comparison with the fragments produced by cleavage of recombinant Na+,K+-ATPase (two right lanes). Cleavage of renal Na+,K+-ATPase in a Na+ containing medium (E1Na(O) conformation) leads to two fragments: 80EWVK and 283HFIH (lanes marked NKA, Na). For the recombinant WT and also the D369N protein in a Na+ containing medium, the two fragments appear in parallel as the Fe2+ concentrations was raised from 10 to 300 μM (Fig. 6, left). By contrast, a comparison of cleavages of WT and D369N mutant in a Rb+ containing medium (E1K(O) conformation) showed a distinct difference (Fig. 6, right). Cleavage of the renal Na+,K+-ATPase in the E1K(O) conformation produces three additional fragments, 214ESE, 608MVTGD, and 712VNDS (lane marked NKA, Rb). Cleavage of the WT recombinant enzyme led to the same three fragments, albeit in reduced yield compared with the renal enzyme. However, the specific cleavages of the E1K(O) conformation were largely suppressed in the D369N mutant. At 10 μM Fe2+ none of the three fragments, 214ESE, 608MVTGD, and 712VNDS, were observed. At high Fe2+ concentrations, 100 and 300 μM, small amounts of the fragments appeared (Fig. 6, right, lanes 6 and 8). These experiments provide strong evidence for two Fe2+ sites in the WT: site 1 (214ESE, 608MVTGD, and 712VNDS) and site 2 (283HFIH), and distortion of Fe2+ binding site 1 by the D369N mutation (see Ref. 14 and “Discussion”).

In optimization experiments, cleavage of the recombinant

|                | WT         | D369N                  |
|----------------|------------|------------------------|
|                | pmol mg protein−1 |  |
| 1 mM Mg2+     | 40.9 (n = 2) | 29.5 (n = 2)          |
| 1 mM Mg2+ + EDTA | 1.23 (n = 2) | 1.01 (n = 2)          |
| 1 mM Fe2+     | 16.1 ± 3.7 (n = 3) | 14.2 ± 1.2 (n = 5) |
| 1 mM Fe2+ + Desferal | 1.06 (n = 2) | 3.25 (n = 2)          |
Fe$^{2+}$-catalyzed Oxidative Cleavage of Recombinant Na$^+$/K$^+$-ATPase

Fe$^{2+}$ and AMP-PNP-Fe$^{2+}$-catalyzed cleavage of WT and D369N Mutant—Fig. 8 presents experiments to test the ATP-Fe$^{2+}$ complex as a substrate for phosphorylation with AMP-PNP-Fe$^{2+}$, on the WT and D369N mutant (see Refs. 11 and 13). The lanes marked Na show the same cleavages as in Fig. 6 (left). In the presence of ATP, the ATP-Fe$^{2+}$ complex is formed and, for the WT, three new fragments are observed (214ESE, near 440VAGDA, and 712VDNS), whereas 263HPFH and 86EWVK fragments are suppressed (lane marked ATP-NA, WT). These features are exactly those described and analyzed previously for cleavage of the renal Na$^+$/K$^+$-ATPase (11, 13). Suppression of the near 263HPFH and near 86EWVK fragments is because of chelation of Fe$^{2+}$ in the ATP-Fe$^{2+}$ complex and reduction of the free Fe$^{2+}$ concentration to a negligible value. The near 440VAGDA and 712VDNS fragments are the product of cleavage mediated by bound ATP-Fe$^{2+}$ acting as an affinity cleavage reagent in the E$_2$-Na-ATP-Fe$^{2+}$ conformation (11, 13). The 214ESE fragment is the product of cleavage of the E$_2$-P conformation, upon phosphorylation by ATP-Fe$^{2+}$ plus Na$^+$ ions, with Fe$^{2+}$ substituting for the Mg$^{2+}$ ion (11). None of these three fragments were observed for the D369N mutant (lane marked ATP-NA, D369N). Cleavage of E$_2$-P can also be observed, without the cleavages mediated by bound ATP-Fe$^{2+}$, by first generating the phosphoenzyme, then arresting phosphorylation with the iron chelator Desferal, and shortly afterward adding the ascorbate/H$_2$O$_2$ (11). When E$_2$-P is isolated kinetically in this way, a major cleavage is seen at the 214ESE position (lane marked ATP-NA Desferal, WT). In the D369N mutant no cleavage is seen (lane marked ATP-NA Desferal, D369N). Lack of cleavage of the D369N mutant, characteristic of the E$_2$-P form, is expected because the mutant cannot undergo phosphorylation. On the other hand, the absence of cleavages mediated by bound ATP-Fe$^{2+}$ was not expected because the D369N mutant is known to bind ATP, and indeed with a much higher affinity than the WT (26).

Figs. 9–11 examine in greater detail specific cleavages of the WT and D369N mutant mediated by the non-hydrolyzable analogue AMP-PNP-Fe$^{2+}$ acting as an affinity cleavage reagent. When bound to the renal Na$^+$/K$^+$-ATPase, the AMP-PNP-Fe$^{2+}$ complex mediates the same cleavages as ATP-Fe$^{2+}$, except for that dependent on phosphorylation (11, 13). In Fig. 9 cleavage was examined in a Na$^+$-containing medium, at 10 or 100 μM Fe$^{2+}$ and 100 μM AMP-PNP. For WT, at 10 μM Fe$^{2+}$ and 100 μM AMP-PNP (maximal AMP-PNP-Fe$^{2+}$, 10 μM plus 90 μM uncomplexed AMP-PNP) near 440VAGDA and 712VDNS fragments were observed, as described previously for the renal Na$^+$/K$^+$-ATPase (lane marked NK1A). By contrast, neither fragment was observed for the D369N mutant, consistent with Fig. 8. The near 263HPFH fragment was not observed because of chelation of the free Fe$^{2+}$ by the AMP-PNP. With 100 μM Fe$^{2+}$ and 100 μM AMP-PNP a large fraction of the nucleotide is in the AMP-PNP-Fe$^{2+}$ form and the concentration of uncomplexed AMP-PNP tends to a low value. For the WT, the near 440VAGDA and 712VDNS cleavages are similar but stronger than at 10 μM Fe$^{2+}$ plus 100 μM AMP-PNP. Strikingly, however, at 100 μM Fe$^{2+}$ and 100 μM AMP-PNP, cleavage of the D369N mutant produced both of the near 440VAGDA and 712VDNS fragments and also a new fragment (asterisk). The new fragment runs parallel to a fragment observed previously (10), with a cleavage position close to the active site aspartate, referred to as near 363CSDK (see lane marked NK2A). The new cleavage designated near 386CSDK in the D369N mutant, seen also in Fig. 10, represents a qualitative difference from the WT.

The appearance of the near 440VAGDA and 712VDNS bands in the D369N mutant only at the higher concentration of AMP-PNP-Fe$^{2+}$ represents a quantitative difference from WT. This
Fe\(^{2+}\)-catalyzed oxidative cleavage of WT and D369N mutant in E,Na or E, (Rb) conformations. Membranes were suspended in 20 mM MES buffer, pH 6.5, and 130 mM NaCl or RbCl was added. Fe\(^{2+}\) concentration was varied as indicated. C, control uncleaved membranes. NKA, pig kidney Na\(^{+},K^{+}\)-ATPase cleaved with Fe\(^{2+}\)/ascorbate/H\(_{2}\)O\(_{2}\) in RbCl or NaCl containing media.

Fig. 7. Oxidative cleavage of WT and D369N mutant in the presence Fe\(^{2+}\), ouabain, without or with Pi. WT and D369N mutant membranes were suspended in 20 mM MES buffer, pH 6.5. Samples were incubated for 20 min on ice in the presence of 500 \(\mu\)M Fe\(^{2+}\), with or without 1 mM phosphate (Tris), and then 0.5 mM ouabain was added and the samples were incubated for a further 15 min. 10 mM Desferal was added and after 5 min 10 mM ascorbate/H\(_{2}\)O\(_{2}\) was added and incubated for 15 min before arresting the cleavage reaction.

The observation could indicate that AMP-PNP-Fe\(^{2+}\) binds less well or is a less effective cleavage reagent on the D369N mutant compared with WT or, alternatively, that uncomplexed AMP-PNP is a much better competitor of the AMP-PNP-Fe\(^{2+}\) complex in the D369N mutant compared with WT, and displaces AMP-PNP-Fe\(^{2+}\) from the enzyme. Both effects could also apply. To distinguish these possibilities, the AMP-PNP concentration was varied systematically from 1 to 5000 \(\mu\)M, namely with AMP-PNP concentrations ranging from far below to far above the Fe\(^{2+}\) concentration (Fig. 10). For both WT and the D369N mutant, as the AMP-PNP concentration was raised the site 2 cleavages (near \(\omega\)EWVK and near \(\omega^8\)HFHI) were progressively suppressed because of chelation of Fe\(^{2+}\), the near \(\omega^4\)VAGDA and \(\omega^7\)VNDS fragments were observed at the intermediate concentrations, and all cleavages were suppressed at the highest AMP-PNP concentrations (1000–5000 \(\mu\)M). Suppression at the high concentrations is because of competitive displacement of AMP-PNP-Fe\(^{2+}\) by uncomplexed AMP-PNP (see Refs. 11 and 13). Fig. 11 represents graphs of the relative amounts of the prominent \(\omega^7\)VNDS fragment for WT and the D369N mutant based on scans. Clearly, the amount of \(\omega^7\)VNDS is significantly lower in the D369N mutant at all concentrations of AMP-PNP. Below 100 \(\mu\)M AMP-PNP, the AMP-PNP-Fe\(^{2+}\) complex is the major species present and at 1–5 \(\mu\)M AMP-PNP the concentration of uncomplexed AMP-PNP must be very low. An approximate \(K_{0.5}\) for the rising phase is 2–3 \(\mu\)M AMP-PNP-Fe\(^{2+}\) for both WT and the D369N mutant. By contrast the \(K_{0.5}\) for the falling phase is much lower for the D369N mutant compared with WT (~300 \(\mu\)M compared with ~3000 \(\mu\)M, respectively). The experiment indicates both that AMP-PNP-Fe\(^{2+}\) mediates cleavages less effectively and also that free AMP-PNP is a better competitor in the D369N mutation.

**DISCUSSION**

*P. pastoris* as an Expression System for Na\(^{+},K^{+}\)-ATPase

The most important feature of the *P. pastoris* system is that rather large amounts of the membranes containing the fully functional recombinant Na\(^{+},K^{+}\)-ATPase, and no endogenous Na\(^{+},K^{+}\)-ATPase activity, can be readily obtained. In addition, the relatively high specific activity observed routinely, ~50 pmol of ouabain binding/mg of protein, and the high yield of membranes, ~300–500 mg/3-liter culture, represent a significant advantage for the cleavage experiments described here and other biochemical work. This site density was achieved by utilizing the protease-deficient strain SMD1165, by screening transformed clones for multicopy integrants of the \(\alpha\) subunit gene and Western blots of the expressed protein, and by opti-
concentration and with ascorbate/H₂O₂.

Fe²⁺ near 367°CSDK.
The ionic strength (Fig. 4).

Membranes were suspended in 20 mM MES buffer, pH 6.5, plus 130 mM NaCl, AMP-PNP was added to the indicated concentration, and membranes were then incubated with Fe²⁺ at the indicated concentration and with ascorbate/H₂O₂. NKA1, pig kidney Na⁺,K⁺-ATPase cleaved in the presence of AMP-PNP-Fe²⁺ (11). NKA2, pig kidney Na⁺,K⁺-ATPase cleaved with Fe²⁺/ascorbate/H₂O₂ at a low ionic strength (Fig. 4C in Ref. 10). The asterisk points to the fragment near 267°CSDK.

FIG. 10. Oxidative cleavage of WT and D369N mutant. Membranes were suspended in 20 mM MES buffer, pH 6.5, plus 130 mM NaCl, AMP-PNP was added to the indicated concentrations, and they were then incubated with 100 μM Fe²⁺ and ascorbate/H₂O₂.

FIG. 11. Quantification of the 267VNDS fragment produced at fixed Fe²⁺ (100 μM) and varied AMP-PNP (1–5000 μM) concentrations. The immunoblot in Fig. 10 was scanned, and the amount of the VNDS fragment in arbitrary units was determined by multiplying the OD by the demarcated area of the band.

Fe²⁺-catalyzed Oxidative Cleavage of Recombinant Na⁺,K⁺-ATPase

By several criteria the expressed protein is functional and structurally intact. The turnover number (about 7500 min⁻¹), calculated from ATPase activities and ouabain binding data, is similar to that of the renal Na⁺,K⁺-ATPase (33). The chymotryptic cleavage data (Fig. 5) show that the recombinant protein can adopt the E₁Na or E₂(K) conformations and expose chymotrypsin-sensitive bonds for selective proteolytic cleavage, as described for the renal enzyme (36). Similarly, oxidative cleavages mediated by bound Fe²⁺ or the ATP-Fe²⁺ complex (Figs. 6–10), indicate that the E₁ ↔ E₂ conformational states, ATP-Fe²⁺ binding, and phosphorylation from ATP-Fe²⁺ are similar to those for the renal Na⁺,K⁺-ATPase (9–14). On the other hand, there are indications that the recombinant protein is less stable than renal Na⁺,K⁺-ATPase, including inactivation by SDS, which precludes purification by the standard SDS/ATP procedure (33), and the necessity to carry out Fe²⁺-cleavage experiments at 0°C rather than at 20°C.

Fe²⁺-catalyzed Oxidative Cleavage

Two Fe²⁺ Sites: A Role for Asp³⁶⁹ in E₁ ↔ E₂ Conformational Changes—In the E₂(Rb) conformation the 214ESE, near 608MVTGD, and 712VNDS fragments cleavages are greatly suppressed by the D369N mutation compared with WT, whereas two other cleavages, the near 258HFIH and near 600EWVK fragments, found in either E₁Na or E₂(Rb) conformations are unaffected (Fig. 6). This finding provides clear evidence for two Fe²⁺ sites: site 1 within the active site (214ESE, near 608MVTGD, and 712VNDS fragments) and site 2 at the membrane-water interface of transmembrane segments M3 and M1 (near 258HFIH and near 600EWVK fragments), as inferred also previously on the basis of less direct evidence (46). The similar cleavages in the presence of either Fe²⁺/Rb or Fe²⁺/ouabain for WT (Figs. 6 and 7) and their suppression in the D369N mutant in both conditions, as well as Fe²⁺-dependent ouabain binding (Table I), now make it clear that Fe²⁺-site 1 represents a Mg²⁺.
binding site in the E2 conformation, in the absence of the substrates ATP or P.

Suppression of site 1 cleavages by the D369N mutation (Figs. 6 and 7) could occur if the mutation acted like Na⁺ ions to stabilize an E1 conformation. However, prior evidence that the D369N mutation stabilizes an E2 conformation (26) shows that the effect has another explanation. The low amounts of the 212TGESE, near 605MVTG, and 712VNDS fragments observed at elevated Fe²⁺ concentrations in Fig. 6 (right) may indicate that Fe²⁺ ions do not bind tightly in the E2(Rb) conformation or that the efficiency of cleavage of the D369N mutant is low. Similarly, lack of cleavage of the D369N mutant in the E2(ouabain) conformation of Fig. 7 indicates either that Fe²⁺ is bound but cannot catalyze oxidative cleavage, or that bound Fe²⁺ dissociates rapidly from the protein and is chelated by Desferal.

Either explanation implies that D369N mutation significantly distorts the Fe²⁺ (Mg²⁺) site 1 in the E₂ conformation.

The previous observation that D369N A mutants stabilize E2 conformations by comparison with the WT enzyme shows that Asp³⁶⁹ plays a role in E₁ ↔ E₂ conformational transitions (26). The present findings allow us to propose such a role for Asp³⁶⁹ in the energetics of the A and P domain interactions, based on the Ca²⁺-ATPase structure. Fe²⁺-catalyzed cleavage of the WT or renal Na⁺,K⁺-ATPase indicated that the E₁Na → E₂(Rb) conformation change brings the 212TGESE sequence (A domain) into close proximity with the 369DKGT and 605MVTG sequences (P domain) (9, 14). The current experiments show that the D369N mutation alters the interaction of Fe²⁺ (Mg²⁺) ions with the 212TGESE (A domain) and 708TGDGVNDS (P domain) sequences in the E₂ conformation, and imply that the relationship between these sequences is altered in the E₂ conformations. Obviously, in the WT enzyme electrostatic repulsion between the charged residues of the TGES sequence (Glu²¹⁴) coming into proximity with those in 369DKGT and 708TGDGVNDS sequences (Asp³⁶⁹ and Asp⁷¹⁰) could hinder the A to P domain interaction, making the E₂ conformation less stabilized than the E₁ conformation in which the A and P domains are separate. In the D369N mutant, removal of the charge could decrease electrostatic repulsions, making the A to P interaction and thus the E₂ conformation energetically more favored. It was proposed previously that neutralization of the charge on the D369N A mutants mimics, at least in part, the bound phosphate and Mg²⁺ ions in the WT enzyme (26). This is an interesting concept because decrease of electrostatic repulsion of the sort just discussed could also trigger the A to P domain movement characteristic of the normal E₁-P ↔ E₂-P transition.

**ATP-Fe²⁺ (Mg²⁺) Binding**—The data in Figs. 9–11 show that cleavage of the D369N mutant with AMP-PNP-Fe²⁺ produces the same fragments 712VNDS (P domain) and near 440VAGDA (N domain) fragments as for WT, but only at elevated AMP-PNP-Fe²⁺ concentrations, and a new cleavage near 367CSDK appears. The effects result from a reduced efficiency of cleavage catalyzed by AMP-PNP-Fe²⁺ and also a raised affinity for AMP-PNP in competing for AMP-PNP-Fe²⁺. The higher AMP-PNP binding affinity is consistent with the known effect of this mutation to raise ATP affinity (26). The findings have two specific implications. First, both of the cleavages at 712VNDS and near 440VAGDA fail to appear at low AMP-PNP-Fe²⁺ and both are restored at elevated AMP-PNP-Fe²⁺. Therefore the mutation provides strong evidence that in the WT the Fe²⁺ in ATP-Fe²⁺ binds simultaneously in the P (D710) and N domains (within 440VAGDA). Second, induction of a new cleavage near the Asp³⁶⁹ itself implies that Fe²⁺ in bound ATP-Fe²⁺ moved toward the 367CSDK sequence.

The effects of the D369N mutation on ATP binding and on cleavage mediated by ATP-Fe²⁺ are explained economically by a recent model of ATP-Mg²⁺ binding to Ca²⁺-ATPase (13), and provide independent evidence for the model. The model is based on the assumption that binding of ATP-Mg²⁺ in an E₁-ATP-Mg²⁺ state induces an N to P domain interaction, in which the γ-phosphate of ATP comes into proximity with Asp³⁵¹ and the Mg²⁺ ion with Asp³⁷³ (P domain) and Glu³⁴⁹ (N domain) (Asp³⁶⁹, Asp⁷¹⁰, and Asp³⁶⁴ for Na⁺,K⁺-ATPase). The assignment of Asp³⁶⁹ of Na⁺,K⁺-ATPase as a Mg²⁺-binding residue is confirmed by mutagenesis work (21), whereas the proposal that Asp³⁶⁴ (Glu³⁴⁹ of Ca²⁺-ATPase) is a Mg²⁺-binding residue was hypothetical, and was made to explain an ATP-Fe²⁺-catalyzed cleavage in the N domain (13). Based on the Fe²⁺-catalyzed oxidation of Thr³⁴⁴ of Ca²⁺-ATPase (Ser³⁴⁵ of Na,K-ATPase) this residue has also been suggested to play a role in Mg²⁺ binding (15). Mutagenesis or chemical modification work have shown that ATP interacts with residues in the N domain including Lys⁵¹⁵, Thr³⁴⁴, Phe³⁸⁷, Arg⁴⁸⁹, and Lys⁴⁹² of Ca²⁺-ATPase and residues Lys⁴⁸⁰, Gly⁴⁹², Lys⁴⁸⁰, and Arg³⁴⁴ of Na⁺,K⁺-ATPase (Lys⁵¹⁵, Gly³⁸⁷, Lys⁴⁹², and Arg³⁶⁹ for Ca²⁺-ATPase) (see Refs. 4 and 47 for reviews). These features appear clearly in the model (13). The large increase in binding affinity for ATP, but not ADP, in D369N A mutants of Na⁺,K⁺-ATPase
(26) or D351N, -A, and -T mutants of Ca\(^{2+}\)-ATPase (30) indicate that neutralization of the charge of the active site aspartate removes repulsion with the \(\gamma\)-phosphate of ATP. Thus, one could hypothesize that the \(\gamma\)-phosphate plus Mg\(^{2+}\) approaches Asn\(^{351}\) or other nearby residues more closely than in the WT, and interacts more strongly.

To test the hypothesis we have calculated possible structural differences between the WT model and D351N by a molecular dynamics simulation, using the published model with bound ATP-Mg\(^{2+}\) as the starting point (13). In the context of the simulation (Fig. 12), it can indeed be observed that, in the model of the mutated form, not only has the distance between the Mg\(^{2+}\) ion and residues in the CDSDKTG sequence shortened significantly (to both Asp\(^{351}\) and Thr\(^{353}\)), but also Thr\(^{353}\) appears to be more accessible to the ion. With Fe\(^{2+}\) replacing Mg\(^{2+}\), this would be compatible with the possibility of the new cleavage in the mutant at Asp\(^{351}\) or Thr\(^{353}\). Mutagenesis of Ca\(^{2+}\)-ATPase suggested that the side chain of Thr\(^{353}\) interacts with \(\beta\)- or \(\gamma\)-phosphates or bridging oxygen of ATP, and the carboxyl group with the Mg\(^{2+}\) ion (48). In both the calculated models of the WT and the Asn\(^{351}\) mutant structures, the Mg\(^{2+}\) ions are depicted as being closely bound to \(\gamma\)- and \(\beta\)-phosphates of ATP, to Asp\(^{353}\) (P domain) and to Glu\(^{359}\) (N domain) (see also Ref. 13).

In the framework of the model structures, differences in the coordination of ATP in the mutated and WT forms are readily detected. Whereas in the WT model, the \(\gamma\)-phosphate of ATP is electrostatically repulsed by the negative charge of Asp\(^{351}\), in the model structure of the mutant, Asn\(^{351}\) stabilizes ATP by hydrogen bonding to the \(\gamma\)-phosphate. Furthermore, based on our simulations, stabilization occurs via Lys\(^{684}\), which in the model of the mutant form is coordinated with the \(\beta\)-phosphate of the ATP, whereas in the model of the WT, Lys\(^{684}\) coordinates Asp\(^{351}\), stabilizing it in a conformation, which keeps it away from the \(\gamma\)-phosphate of ATP. Thus, within the framework of the presented calculations, the observed increase in binding affinity upon D351N mutation can be explained by three contributions: first, loss of electrostatic repulsion between Asp\(^{351}\) and ATP; second, gain of hydrogen bonds between Asn\(^{351}\) and the \(\gamma\)-phosphate; and third, further electrostatic stabilization of the ATP by Lys\(^{684}\).

It was speculated earlier that mutations of the Asp\(^{351}\) of Ca\(^{2+}\)-ATPase to either A or N or T induce a conformational analogy to the phosphorylation transition state (30). In our WT model, the distance between the active site, Asp\(^{351}\) and the \(\gamma\)-phosphate is on the order of 8 Å, whereas in the model of the mutant the distance is significantly shortened, on the order of 3.5 Å. Thus the models of the wild type and the mutant support the notion that the latter might resemble a transition state analogue. In this context, one can also speculate that the loss of electrostatic attraction between Asp\(^{351}\) and Lys\(^{684}\), which will have to occur to allow Asp\(^{351}\) to approach the \(\beta\)-phosphate of the ATP, might be compensated by binding of Lys\(^{684}\) to the \(\beta\)-phosphate of ATP, thus lowering the activation energy of phosphorylation. The model of the mutant structure (with Asp\(^{351}\) instead of Asn\(^{351}\)) could resemble an E\(_{1}\)-ATP-Mg\(^{2+}\) state with 2Ca\(^{2+}\) ions bound and ready to phosphorylate Asp\(^{351}\), whereas the model of the WT structure would be analogous to an E\(_{1}\)-ATP-Mg\(^{2+}\) form in the absence of Ca\(^{2+}\) ions, and unable to phosphorylate Asp\(^{351}\). The equivalent conformations of Na\(^{+}\),K\(^{+}\)-ATPase would be E\(_{1}\)(3Na\(^{+}\))ATP-Mg\(^{2+}\) with 3Na\(^{+}\) ions bound and poised for phosphorylation (mutant structure), or E\(_{1}\)-ATP-Mg\(^{2+}\) with ATP bound tightly but still unable to phosphorylate (WT structure), respectively.

The work described here shows how the Na\(^{+}\),K\(^{+}\)-ATPase expressed in P. pastoris can be utilized to study events in the active site by Fe\(^{2+}\)-catalyzed oxidative cleavage. The recombiant Na\(^{+}\),K\(^{+}\)-ATPase in P. pastoris appears to provide a promising tool for studying the energy transduction mechanism by Fe\(^{2+}\)-catalyzed oxidative cleavages, or other techniques, particularly when combined with molecular modeling.
Expression of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in Pichia pastoris: ANALYSIS OF WILD TYPE AND D369N MUTANT PROTEINS BY FE2+-CATALYZED OXIDATIVE CLEAVAGE AND MOLECULAR MODELING

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J. Biol. Chem. 2003, 278:46064-46073.
doi: 10.1074/jbc.M308303200 originally published online August 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308303200

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