Role of Phylogenetically Conserved Amino Acids in Folding of Na,K-ATPase[†]

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ABSTRACT: This paper focuses on the amino acid sequence 708-TGDGVNDSPALKK in pig kidney Na,K-ATPase as one of the best conserved among P-type ATPases. In Ca-ATPase this sequence forms a strand−loop−helix structure as part of a Rossman fold next to the phosphorylation site. Substitution of polar residues in the investigated sequence interfered with high-level accumulation of mutant protein. Mutant α_1-subunit protein only accumulated in membranes from yeast cells grown at 15 °C whereas wild-type protein accumulated at both 15 and 35 °C. A systematic screen for the molecular mechanism behind lack of accumulation of mutant protein at 35 °C showed that transcription and translation were unaffected by the mutations. To demonstrate in vivo protein folding problems, an unfolded protein response reporter system was constructed in yeast. In this strain, only expression of mutant Na,K-ATPase α_1-subunit caused induction of the unfolded protein response at 35 °C, indicating folding problems in the ER. Lowering the expression temperature to 15 °C prevented induction of the unfolded protein response after mutant protein expression, indicating correct folding at this temperature. At the permissive temperature mutant proteins were able to escape the endoplasmic reticulum quality control, reach the plasma membrane, and bind ouabain with high affinity. Since mutants in the 708-TGDGVNDSPALKK segment had a thermo inactivation profile identical to that of wild type, they were classified as temperature-sensitive synthesis mutants. The results indicate that this segment contributes side chains of importance for overall folding and maturation of Na,K-ATPase and all other P-type ATPases.

Kidney Na,K-ATPase, a member of the P-type ATPase superfamily, is an $\alpha_1\beta_1$ heterodimer consisting of an α_1-subunit of 1016 residues and a 302 residue long β_1-subunit. Biochemical work has demonstrated that the α_1-subunit has 10 transmembrane segments while the β_1-subunit is a single span membrane protein with three disulfide bonds and three N-glycosylation sites (*1*−*4*). The α_1-subunit is the catalytic subunit while the presence of the β_1-subunit protects the α_1-subunit from intracellular degradation (*5*).

Conservation of primary structure among members of a protein family is of functional importance (*6*). P-Type ATPases contain eight amino acid sequences that are conserved among members of this superfamily (*7*, *8*). The conserved residues may contribute side chains for interactions with ligands common to all P-type ATPases or for conformational transitions (*8*, *9*). Experiments showing that biosynthesis of yeast PMA1[1] H-ATPase can be disrupted by single amino acid substitutions in the transiently phosphorylated residue Asp-378 (*10*, *11*) or neighboring residues (*12*) have implicated a role for phylogenetically conserved amino acids in folding of P-type ATPases.

The sequence shown in Table 1 is one of the phylogenetically best conserved among known P-type ATPases (*7*, *8*). In the high-resolution structure of the homologous SR Ca-ATPase, this sequence forms a strand−loop−helix located in the P-domain with connections to M5 (*14*). Residues in this conserved region have recently been shown to contribute to coordination of Mg^{2+} and phosphoryl transfer in the E_1 conformation of the Na,K-ATPase and to vanadate or phosphate binding in the E_2 conformation (*15*).

For polytopic proteins, folding, biogenesis, and particularly control of membrane insertion are still poorly understood. The current view is that membrane protein topogenesis is governed by alternating signal anchor and stop transfer sequences (*16*). For Na,K-ATPase it has been shown in vivo and in vitro that topogenesis of the four hydrophobic N-terminal transmembrane segments follows the general model as M1 and M3 exhibit efficient signal anchor activity while M2 and M4 show efficient stop transfer activity (*17*−*19*). Membrane insertion of the C-terminal part of Na,K-ATPase is, however, more complicated. M5 has poor topogenic activity, and posttranslational hairpin formation with M6 may be required for efficient membrane insertion (*17*, *20*). With the exception of M10, the six C-terminal transmembrane segments all have poor membrane insertion properties, but interactions of the loop between M7 and M8 with the β-subunit may promote membrane insertion (*20*). Topogenesis of the C-terminal part of the Na,K-ATPase can therefore not be fully explained by alternating signal anchor and stop transfer sequences (*21*). Specific sequences upstream and/or downstream of individual transmembrane segments

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[1] Abbreviations: PMA1, yeast H-ATPase; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; M, membrane segment; SD, standard deviation; UPR, unfolded protein response; UPRE, unfolded protein response element; MDCK, Madin-Darby canine kidney cells.

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Table 1: Alignment of One of the Best Conserved Sequences in P-Type ATPases[a]

ATPase	Sequence	Conserved
	*	
Na,K- and H,K-ATPase	708 TGDGVNDSPALKK 720	(37/44)
Ca-ATPase (SR)	TGDGVNDAPALK-	(53/58)
Ca-ATPase (PM)	TGDGTND-PALK-	(18/26)
H-ATPase	TGDGVNDAP-LKK	(32/39)
Consensus	**TGDG-ND-P-LK-**	(140/167)
	↓ ↓ ↓↓↓ ↓↓	
Mutants constructed	A A AAA AA	
	S N QNT RR	

[a] The numbers of identical sequences out of the total number of known sequences for a particular ATPase are shown in parentheses. The consensus sequence and mutants constructed are shown at the bottom. *: from the submitted α_1-pig cDNA sequence (*13*), amino acid 718 is a serine. Sequencing this cDNA in our laboratory revealed a leucine at this position as in the other P-type ATPases. Amino acids are numbered according to pig kidney Na,K-ATPase. Data from http://Biobase.dk/~Axe/Patbase.htlm.

M5−M10 may consequently play important roles for folding.

We have consistently produced a large number of enzymes with mutations either in transmembrane segments (*22*−*24*) or in the phosphorylation site (*25*, *26*) in *Saccharomyces cerevisiae* at temperatures between 30 and 35 °C. However, mutation of polar residues in 708-TGDGVNDSPALKK (Table 1) prevented high-level accumulation of mutant protein at these temperatures. The purpose of this study has been to identify the molecular mechanism behind this lack of accumulation and investigate the role of the conserved segment in Na,K-ATPase folding. Thermosensitive accumulation of mutant protein was demonstrated by western blotting and ouabain binding to crude membranes from yeast cells grown at different temperatures. Steady-state mRNA levels were analyzed by northern slot blotting to investigate if reduced accumulation of mutant protein at 35 °C was due to reduced mRNA synthesis or stability. Immunoprecipitation was used to investigate the translation of mutant and wild-type mRNA. The next step in the biosynthetic pathway of Na,K-ATPase is insertion in the ER membrane and folding. An integrated part of the ER quality control system (*27*) is the unfolded protein response, a signaling pathway from the ER lumen to the nucleus that allows eucaryotic cells to respond to the presence of unfolded proteins in the ER (*28*). Activation of the unfolded protein response increases transcription of genes encoding ER-located molecular chaperones and enzymes involved in ER-associated degradation (*29*−*31*). To detect in vivo folding problems of mutant Na,K-ATPase α_1-subunits in the ER, an unfolded protein response reporter system was constructed. Cellular targeting of mutant and wild-type protein was monitored by measuring the number of [^{3}H]ouabain sites exposed on intact yeast cells relative to the number of [^{3}H]ouabain sites in yeast total membranes.

Temperature-sensitive mutations can be divided into two classes: thermolabile and temperature-sensitive synthesis mutants (*32*). Thermolabile mutant proteins produced at the permissive temperature are inactivated on exposure to the restrictive temperature. In temperature-sensitive synthesis mutants, the protein folding pathway is interrupted, and defects are only observed if the protein is synthesized at the restrictive temperature. Mutant thermoinactivation profiles identical to wild type were used to categorize Na,K-ATPase mutations as temperature-sensitive synthesis mutants.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Site-directed mutagenesis of residues T708, N713, S715, K719, and K720 was performed with double-stranded oligonucleotides carrying single codon alterations. The annealed oligo's which have *Ehe*I−*Nco*I sticky ends were cloned into similarly digested pUC19 (*33*) carrying a 1321 bp *Aat*II−*Hin*dIII fragment from pig α_1-cDNA. α_1-Subunit cDNA with the desired mutations was excised as an *Aat*II−*Hin*dIII fragment and inserted into the expression plasmid pPAP1666 (*25*). Site-directed mutagenesis of residues D710 and D714 was done according to Ho et al. (*34*). PCR fragments containing mutations were subsequently cloned into the expression plasmid pPAP1666 (*25*). The nucleotide sequence of all constructs was confirmed by dideoxy sequencing.

Plasmid Constructions. The plasmid pPAP2540 carrying a transcriptional fusion between the *Escherichia coli lacZ* gene and a crippled CYC1 promoter fused to an unfolded protein response element was constructed by exchanging the LEU2 marker present in pJC3 (*35*) with a LYS2 marker. Plasmid pJC3 was a generous gift from Dr. Peter Walter, UCLA, San Francisco, CA.

Yeast Strains. To be able to monitor in vivo folding problems in the ER, pPAP2540 was integrated into the *lys2* allele of PAP1500 (*25*) by homologous recombination.

Transformation of Yeast Cells. Yeast cells were transformed as described by Gietz et al. (*36*).

Small-Scale Production of Recombinant Na,K-ATPase. Yeast cells were grown in shake flasks in minimal medium containing 0.5% glucose and 2% glycerol as carbon source and supplemented with all amino acids except leucine, isoleucine, tryptophan, and histidine. Cultures were grown at 30−35 °C until $OD_{450} = 1.0$, transferred to the selected temperature, and induced with 2% galactose after 30 min thermo equilibration.

Large-Scale Production of Recombinant Na,K-ATPase. Yeast cells were grown in a 10 L jacketed Applicon fermentor equipped with an ADI 1030 Bio Controller connected to a computer running BioXpert 1.10.024 from BioExpert Ltd. Production of wild-type Na,K-ATPase was done at 30 °C as before (*25*). Yeast cells carrying expression plasmids for mutations in the conserved 708-TGDGVNDSPALKK segment were grown at 30 °C until all glucose was metabolized as described before (*25*). The yeast culture was then cooled to 15 °C by circulating water from a temperature-regulated water bath in the fermentor jacket. After 30 min temperature equilibration the culture was induced with 2% galactose. After 48 h growth at 15 °C yeast cells were harvested by centrifugation.

Equilibrium [^{3}H]Ouabain Binding to Yeast Membranes and Intact Yeast Cells. Yeast membranes (100−200 μg) were incubated at 37 °C for 1 h in 3 mM $MgSO_4$, 1 mM Tris−vanadate, 1 mM EGTA, 10 mM MOPS−Tris, pH 7.2, in the presence of 15 nM [^{3}H]ouabain and varying concentrations of cold ouabain and 1 mM PMSF, 1 μg/mL chymostatin, pepstatin, and leupeptin (*25*, *26*). After the mixture

was allowed to stand at 4 °C for 20 min, bound and unbound ouabain were separated by centrifugation at 265000*g* for 30 min at 4 °C. Samples were washed twice in ice-cold binding buffer and centrifuged at 265000*g* for 10 min. The amount of bound [^{3}H]ouabain was determined by scintillation counting. Binding to intact yeast cells were done with 10 mg of yeast using the same protocol as for crude membranes except that the binding buffer contained 1 M sorbitol and centrifugation was at 2000*g*. Lines were fitted by nonlinear least-squares regression using SigmaPlot 2000 from Jandel Scientific to the Michaelis−Menten equation: ouabain binding = a[ouabain]/c + [ouabain], where a is the maximum binding and c is the dissociation constant, K_D.

Thermal Stability of Recombinant Na,K-ATPase. Crude yeast membranes (200 μg) resuspended in 300 μL of lysis buffer (25 mM imidazole, 1 mM EDTA, 1 mM EGTA, 10% sucrose, pH 7.5) containing 1 mM PMSF and 1 μg/mL leupeptin, chymostatin, and pepstatin were incubated in a water bath at 37, 40, 43, 46, 49, or 52 °C for 90 s. Membranes were placed on ice for 15 min, and the amount of ouabain bound at 37 °C was measured at 15 nM [^{3}H]-ouabain. Lines were fitted by nonlinear least-squares regression using SigmaPlot 2000 from Jandel Scientific to the Hill equation with three parameters: ouabain (%) = $ax^b/(c^b + x^b)$, where a is maximum ouabain bindning, b is the Hill coefficient, c is $T_{1/2}$, and x is the temperature.

RNA Isolation and Slot Blot Analysis. Total yeast RNA was isolated with the RNeasy Midi kit from Quiagen (Quiagen Inc.). α_1 pig, β_1 pig, and yeast actin specific probes were generated by PCR and labeled with [α-^{32}P]ATP using the High Prime labeling kit from Roche Chemicals. The α_1 probe contains nucleotides 1−3280, the β_1 probe contains nucleotides 167−1012, and the yeast actin probe carries nucleotides −971 to +1453 all calculated from nucleotide A in the initiation codon. Slot blot analysis was performed with 10 μg of total RNA according to the protocol described by GeneScreen (NEN Research Products). Results were quantified on a STORM 840 PhosphorImager with the ImageQuaNT 4.2a software package from Molecular Dynamics.

SDS−PAGE and western blotting were performed as previously described (*25*) except that western blots were quantified on a STORM 840 PhosphorImager with the ImageQuaNT 4.2a software package from Molecular Dynamics. A polyclonal rabbit anti-α_1-antibody was raised against the recombinant large cytoplasmic loop of α_1 Na,K-ATPase from pig and affinity purified prior to use. The antigen was produced with a histidine tag in *E. coli*. Anti-H-ATPase antibodies were a generous gift from Dr. Michael Palmgren, Royal Veterinary and Agricultural University, Denmark.

Labeling and Immunoprecipitation of Na,K-ATPase. Yeast cells were grown in 50 mL of minimal medium containing 0.5% glucose and 2% glycerol supplemented with all amino acids except leucine, isoleucine, tryptophan, histidine, cysteine, and methionine. Cultures were grown at 35 °C until OD_{450} = 1.0 and induced with 2% galactose. After 4 h 100 μCi of PRO-MIX [^{35}S] cell labeling mix was added (Amersham Pharmacia Biotech). Labeling was stopped after an additional 1 h by addition of sodium azide to a final concentration of 10 mM. Yeast cells were harvested and crude membranes prepared. Immunoprecipitiation with polyclonal anti-α_1-antibody against the large cytoplasmic domain of Na,K-ATPase was performed as described before (*37*). Precipitated proteins were separated by SDS−PAGE; the gel was dried and analyzed on a STORM 840 PhosphorImager.

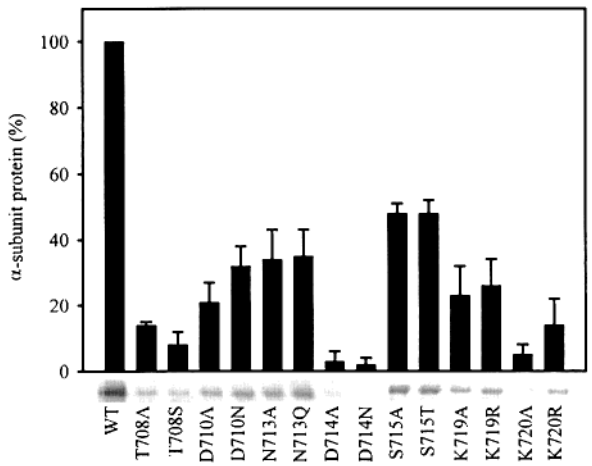

FIGURE 1: Accumulation of wild-type or mutant α_1-subunit protein in crude membranes from yeast cells grown at 30−35 °C. Yeast cells carrying expression plasmids for wild-type or mutant Na,K-ATPase were grown in shake flasks in a thermo room at 30−35 °C until OD_{450} = 1.0, induced with 2% galactose, and harvested 48 h later. Crude membranes were isolated and equal amounts of total protein analyzed by western blotting with anti-α_1-antibodies. Blots were quantified on a PhosphoImager with the ImageQuaNT 4.2a software and wild-type expression defined as 100%. Bars represent mean value ± SD for each mutant relative to wild type determined from three independent growth experiments. The western blot represents one out of three experiments.

Measurements of β-galactosidase activity were performed according to ref *38*.

RESULTS

Mutations to 708-TGDGVNDSPALKK Are Poorly Expressed in Yeast. Several mutants in pig kidney Na,K-ATPase α_1-subunit have previously been constructed and coexpressed with wild-type β_1-subunits in our yeast expression system (*22*−*26*). The same expression protocol was followed for yeast cells transformed with expression plasmids carrying wild-type Na,K-ATPase or the mutations shown in Table 1. Data in Figure 1 show that membranes isolated from these mutants have protein expression levels between 3% and 48% compared to wild type.

Temperature-Dependent Accumulation of Wild-Type, N713A, and S715A Enzymes. To test whether accumulation of mutant protein was temperature sensitive, wild type and two representative mutants, α_1(N713A)β_1 and α_1(S715A)β_1, were expressed at temperatures ranging from 15 to 35 °C. The results of ouabain binding and western blotting on crude membranes isolated from these yeast cells are shown in Figure 2A,B. For wild type it can be seen that the density of ouabain sites and α_1-subunit protein remained high irrespective of expression temperature. Increasing the temperature above 15 °C reduced the density of ouabain sites and α_1-subunit protein to the same extent for the α_1(N713A)β_1 and α_1(S715A)β_1 enzymes.

To study this temperature dependency in further detail, we determined the time-dependent accumulation of ouabain sites in crude membranes from yeast expressing wild-type $\alpha_1\beta_1$, α_1(N713A)β_1, or α_1(S715A)β_1 Na,K-ATPase at 15 and

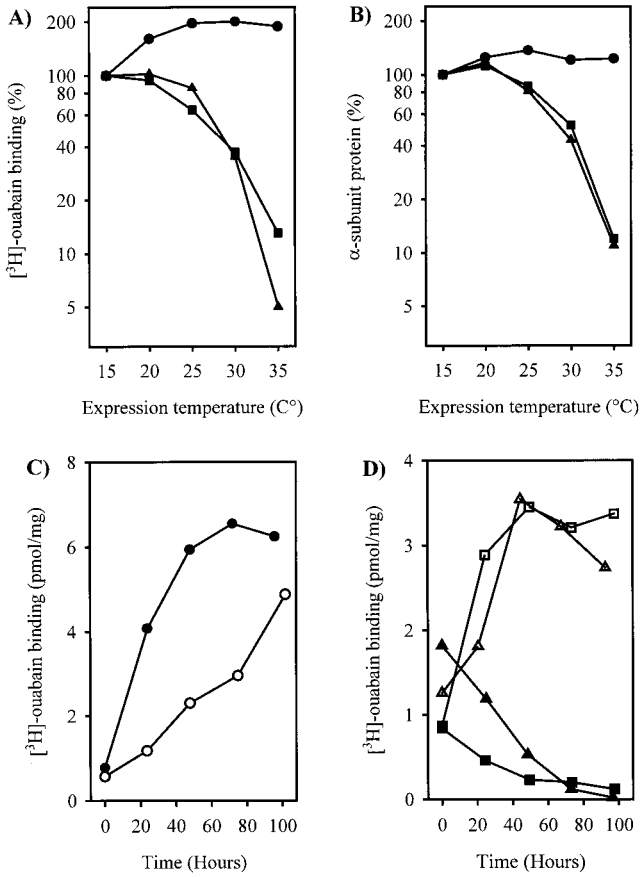

FIGURE 2: (A, B) Effect of expression temperature on accumulation of ouabain sites (A) and α_1-subunit protein (B) in crude membranes in yeast expressing wild-type $\alpha_1\beta_1$ (●), α_1(N713A)β_1 (■), or α_1(S715A)β_1 (▲). Yeast cells were grown at 30 °C until OD_{450} = 1.0, and aliquots were shifted to 15, 20, 25, 30, or 35 °C. Na,K-ATPase biosynthesis was initiated by addition of 2% galactose after temperature equilibration (30 min). After 48 h membranes were prepared, and ouabain binding was performed in the presence of 15 nM [^{3}H]ouabain. The amount of α_1 protein was determined by western blotting on 10 μg of total membrane protein. The density of ouabain sites and α_1 protein present at 15 °C was defined as 100%. Data represent one out of two experiments. (C, D) Time-dependent accumulation of ouabain sites at 15 and 35 °C in crude membranes from yeast carrying wild-type $\alpha_1\beta_1$ (C, ●, ○), α_1(N713A)β_1 (D, ■, □), or α_1(S715A)β_1 (D, ▲, △) expression plasmids. Yeast cells were grown at 35 °C until OD_{450} = 1. One portion of cells was induced with 2% galactose and continued growth at 35 °C (closed symbols). Another portion was shifted to 15 °C and induced with 2% galactose after 30 min (open symbols). At the times indicated samples were harvested by centrifugation and stored at −80 °C. Crude membranes were isolated for all samples, and ouabain binding was performed in the presence of 15 nM [^{3}H]ouabain. Data represent one out of two or three independent growth experiments of each yeast strain.

35 °C. It can be seen from Figure 2C that the density of wild-type enzyme increased both at 15 °C and at 35 °C after galactose induction. In contrast, there was a decrease in the density of high-affinity ouabain sites in yeast cells induced to express α_1(N713A)β_1 or α_1(S715A)β_1 at 35 °C (Figure 2D). The pool of mutant Na,K-ATPase present before induction was absent after 3 days at 35 °C. However, after induction at 15 °C, α_1(N713A)β_1 and α_1(S715A)β_1 units accumulated to the same extent. Accumulation peaked after 48 h. Therefore accumulation of α_1(N713A)β_1 and α_1-(S715A)β_1 is temperature sensitive.

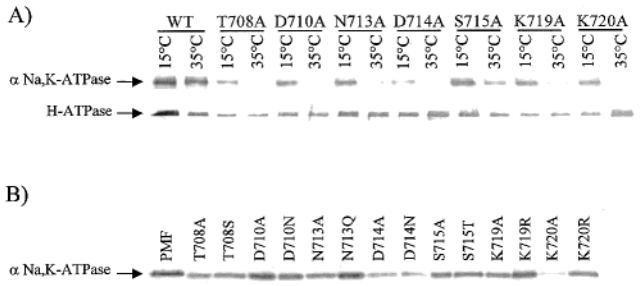

FIGURE 3: Temperature-dependent accumulation of mutant Na,K-ATPase. (A) Yeast cells carrying a wild-type $\alpha_1\beta_1$ or mutant Na,K-ATPase were grown as described in Figure 2C,D and harvested after 48 h. Crude membranes were isolated and equal amounts of total membrane protein analyzed by western blotting with anti-α_1-antibodies or anti-H-ATPase antibodies as indicated. Western blots represent one out of three experiments. (B) Accumulation of α_1-subunit protein in crude yeast membranes isolated 48 h after cells were shifted to 15 °C and induced with 2% galactose. Membrane proteins (10 μg) were separated by SDS−PAGE and developed with anti-α_1-antibodies. The lane used for application of each α_1-subunit mutant is indicated. PMF: pig kidney microsomal fraction. The western blot represents one out of three experiments.

Temperature-Dependent Accumulation of All Mutants in the T708−K720 Region. To analyze whether alteration of any polar conserved amino acids in the T708−K720 region caused temperature-sensitive accumulation, the density of α_1-subunit protein present in crude membranes from all alanine mutants induced at either 15 or 35 °C was determined by western blotting (Figure 3A). In contrast to wild type, all mutant proteins accumulated to a considerably higher density at 15 °C than at 35 °C. Very little or no mutant protein could be detected for T708A, D710A, N713A, D714A, K719A, and K720A at 35 °C. This temperature-dependent accumulation was specific for mutant Na,K-ATPase since the endogenous H-ATPase was expressed in comparable amounts at 15 and 35 °C. The results in Figure 3B show that all alanine and conservative mutant proteins except D714A/N and K720A accumulated to comparable levels at 15 °C.

mRNA Levels at 35 °C Are Identical for α_1(wt)β_1 and α_1(S715A)β_1. To test whether the observed temperature-sensitive accumulation of mutants was due to reduced mRNA levels at high temperature, northern slot blots were performed on total RNA isolated from yeast cells expressing wild-type $\alpha_1\beta_1$ or α_1(S715A)β_1 alleles at 35 or 15 °C. Each RNA preparation was probed with an α_1-mRNA specific probe, a β_1-mRNA specific probe, and an actin-mRNA specific probe.

From Figure 4 it is seen that accumulation of α_1-mRNA at different time points after induction was similar for wild type and S715A at 15 and 35 °C, respectively. Northern slot blotting with a β_1 probe showed that accumulation of β_1-mRNA was independent of the coexpressed α_1 allele (data not shown). These results show that mRNA synthesis and stability were unaffected by the mutation. Therefore, the observed differences in α_1-protein accumulation between wild type and the representative mutant α_1(S715A)β_1 must have its origin at the posttranscriptional level.

Mutant Protein Is Synthesized at 35 °C. Immunoprecipitation of wild type and all alanine substitutions was carried out to investigate whether disappearance of mutant protein at 35 °C was due to defective translation. From Figure 5 it is seen that both wild-type and alanine-substituted Na,K-ATPase were synthesized at 35 °C. Therefore, reduced

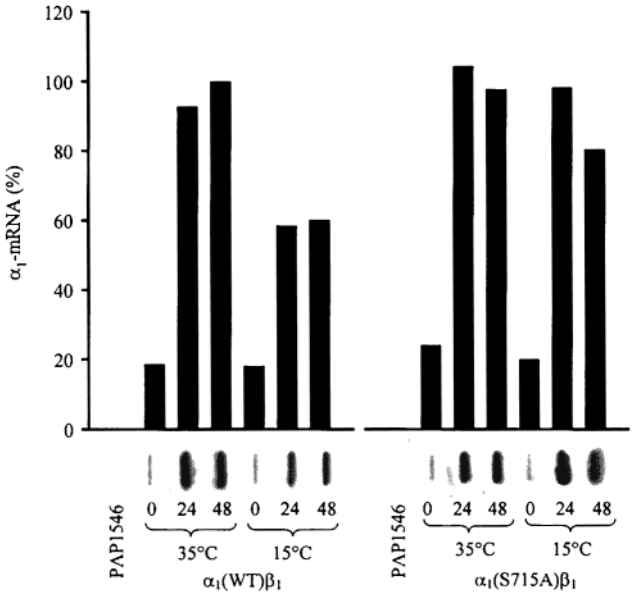

FIGURE 4: Accumulation of α_1-mRNA in yeast cells expressing wild-type $\alpha_1\beta_1$ or α_1(S715A)β_1 Na,K-ATPase. Yeast cells carrying a wild-type $\alpha_1\beta_1$, α_1(S715A)β_1, or an empty expression vector (PAP1546) were grown at 35 °C until $OD_{450} = 1$ ($t = 0$). One portion of cells was induced with 2% galactose and continued growth at 35 °C. Another portion was shifted to 15 °C and induced with 2% galactose after 30 min. In the figure, time points (hours) for RNA isolation are shown below the slots together with growth temperature (°C) and the expressed allele. A total of 10 μg of total RNA was loaded in each slot. Quantification of these data is shown above the slots. Blots were quantified on a PhosphorImager with the ImageQuaNT 2.4 software package from Molecular Dynamics. After normalization of all data to an actin blot (not shown in the figure) the scan value of the empty expression plasmid was subtracted from all samples and the maximum wild-type value defined as 100%. Data were obtained from the same blots.

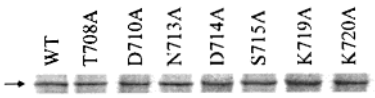

FIGURE 5: Protein synthesis of wild-type and alanine substitutions at 35 °C. Yeast cells producing wild-type or mutant Na,K-ATPase were grown at 35 °C to $OD_{450} = 1$ and induced with 2% galactose. After 4 h the culture was pulsed with [^{35}S]cysteine/methionine for 1.5 h and Na,K-ATPase immunoprecipitated and analyzed as described in Experimental Procedures. The arrow indicates the position of Na,K-ATPase α_1-subunits.

accumulation of mutant protein must have its origin at the posttranslational level.

Alteration of the 708-TGDVNDSPALKK Sequence Affects Protein Folding in the ER. To monitor in vivo folding problems of mutant and wild-type proteins in the ER, we integrated into our yeast strains a transcriptional fusion between the *E. coli lacZ* gene and a crippled CYC1 promoter fused to an unfolded protein response element. Figure 6 shows that expression of mutant protein at 35 °C in this reporter yeast strain increased the unfolded protein response between 3- and 45-fold compared to the response after expression of wild-type $\alpha_1\beta_1$. In contrast, expression at 15 °C almost abolished the unfolded protein response. The K720R mutant is special since its expression induces a very high unfolded protein response at 35 °C and a high response at 15 °C. The temperature-dependent difference in unfolded protein response is tightly correlated to the reduced accumulation of all substitutions at 35 °C compared to 15 °C.

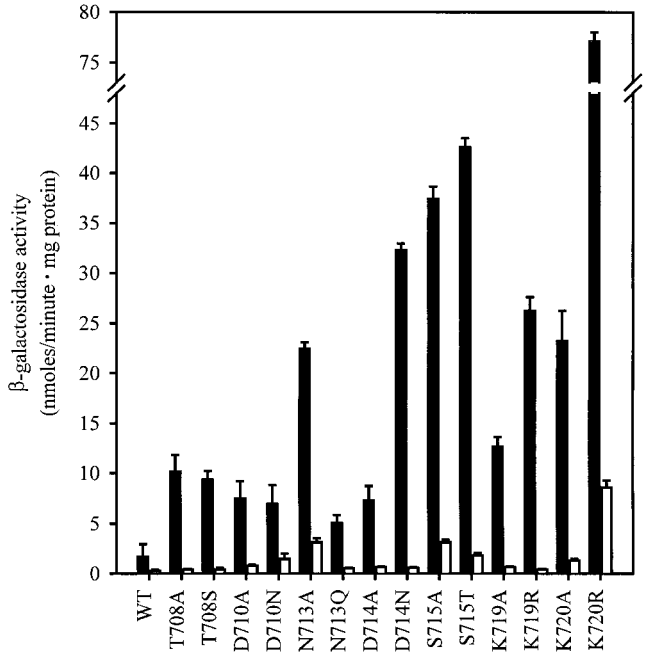

FIGURE 6: Induction of the unfolded protein response. Yeast cells carrying the UPRE-*LacZ* reporter fusion and wild-type Na,K-ATPase, mutant Na,K-ATPase, or an empty expression plasmid were grown at 35 °C to $OD_{450} = 1$. Half of each culture was induced with 2% galactose and continued growth at 35 °C (■) whereas the other half was transferred to 15 °C and induced after 30 min (□). After 48 h specific β-galactosidase activity [nmol/(min·mg of protein)] was determined in purified cytoplasmic fractions according to Rose and Botstein (*38*). The values have been corrected for background activity by subtracting the β-galactosidase activity determined for the empty expression vector. Data are given as mean values ± SD from two independent growth experiments with double determinations of each data point.

This identifies folding in the ER as the temperature-sensitive step in the synthesis of Na,K-ATPase with mutations in the conserved 708-TGDGVNDSPALKK segment.

Mutant α-Subunits Reach the Plasma Membrane at 15 °C. The ratio of yeast cell surface expression to that in crude membrane preparations was determined by ouabain binding to examine if mutant protein produced at 15 °C is retained in the ER. This ratio is relevant for identification of possible intracellular pools since newly assembled Na,K-ATPase catalyzes Mg^{2+} and P_i stimulated ouabain binding (*39*) and all mutants except D714 substitutions bind ouabain with high affinity (*15*). From Figure 7 it is seen that this ratio for mutant proteins ranges from 0.5 to 2.2 compared to 2.0 for wild type. Variations among the ratios are small compared to what would be expected if mutant protein only accumulated in intracellular karamellae structures as observed in yeast in response to expression of some P-type ATPases (*40*, *41*). It is therefore concluded that mutant protein produced at the permissive temperature is able to escape the ER and reach the plasma membrane to an extent similar to that of wild-type Na,K-ATPase produced at 30 °C, the traditional temperature for large-scale fermentation.

Alanine Substitutions Show Temperature Inactivation Profiles Comparable to Wild-Type. To characterize the temperature-sensitive Na,K-ATPase mutants, we studied the temperature inactivation profile for all alanine substitutions produced at 15 °C. Data in Figure 8 and Table 2 show that all alanine substitutions had the same resistance/sensitivity

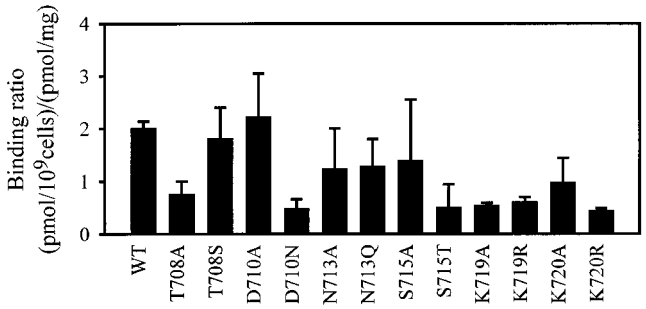

FIGURE 7: Ratio of surface expression to that in crude membranes for wild-type and mutant Na,K-ATPase as determined by [^{3}H]-ouabain binding. Wild-type Na,K-ATPase was produced with the traditional fermentation procedure at 30 °C (*25*). Mutant Na,K-ATPase was produced by large-scale computer controlled fermentation at 15 °C as described in Experimental Procedures. Capacities for ouabain binding to intact cells and crude membranes were calculated from three independent binding experiments. Data are absent for mutations to D714 since they do not bind ouabain (*15*). Data are given as mean values ± SD.

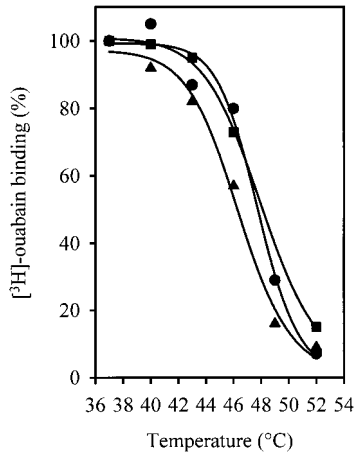

FIGURE 8: Temperature inactivation of wild-type (●), N713A (■), and S715A (▲) Na,K-ATPase. Inactivation was determined as recovery of [^{3}H]ouabain sites after 90 s exposure to 37, 40, 43, 46, 49, or 52 °C. The density of ouabain sites present after 90 s exposure to 37 °C is defined as 100%. $T_{1/2}$ is the temperature (°C) at which half of the ouabain sites present at 37 °C has been inactivated. Data represent one out of two experiments.

Table 2: Temperature Inactivation of Wild-Type and Mutant Na,K-ATPase[a]

allele	$T_{1/2}$ (°C)	allele	$T_{1/2}$ (°C)
WT	47.7 ± 0.8	S715A	43.9 ± 0.3
	48.6 ± 0.2		46.4 ± 0.3
T708A	48.4 ± 0.2	K719A	49.6 ± 0.4
	49.9 ± 0.2		45.8 ± 0.4
D710A	49.2 ± 0.6	K720A	48.1 ± 0.3
	48.6 ± 0.3		48.6 ± 0.2
N713A	49.7 ± 0.4		
	47.4 ± 0.1		

[a] $T_{1/2}$ is the temperature (°C) at which half of the ouabain sites present at 37 °C has been inactivated. Inactivation as described in Figure 8. Data from two independent experiments.

to thermoinactivation as wild-type Na,K-ATPase produced at 30 °C. This shows that lack of accumulation of mutant protein at 35 °C was not due to thermodynamic instability of the final folded conformation. Accordingly, all mutants can be categorized as temperature-sensitive synthesis mutants.

DISCUSSION

The starting point for this study was the observation that accumulation of mutant Na,K-ATPase to a level that allows biochemical characterization was not possible at 30−35 °C for proteins with amino acid alterations in the conserved 708-TGDGVNDSPALKK segment. We systematically investigated possible molecular mechanisms behind lack of accumulation of mutant protein and found transcription and translation to be intact for selected mutants. However, mutants selectively induced the unfolded protein response at 35 °C, indicating folding problems in the ER. Lowering the temperature to 15 °C abolished or dramatically reduced the unfolded protein response, in agreement with accumulation of mutant protein in the plasma membrane at this temperature. Proper folding of mutant protein produced at 15 °C was demonstrated by their ability to bind ouabain and ATP (*15*) with high affinity. This indicates that polar amino acids in one of the phylogenetically most conserved regions among P-type ATPases contribute side chains of importance for overall folding and maturation of Na,K-ATPase.

Since H-ATPase and wild-type Na,K-ATPase accumulated both at 15 °C and at 35 °C, thermosensitive accumulation was specific for mutant Na,K-ATPase (Figure 3). Low expression of mutant α_1 protein at 35 °C, as shown in Figures 1 and 2, could theoretically originate from a reduced steady-state mRNA level caused by a reduced transcription initiation rate or increased mRNA degradation. However, northern slot blots in Figure 4 show that the α_1-mRNA density for the representative mutation, S715A, was similar to the wild-type α_1-mRNA level under identical conditions. Therefore, the lack of α_1-subunit accumulation at 35 °C should find its explanation at the posttranscriptional level. As data in Figure 5 show that all alanine mutant α_1 protein was synthesized at 35 °C, degradation of mutant protein after galactose induction is the most likely explanation for the severely reduced protein levels observed at elevated temperature. At 35 °C mutant α_1-subunits may get trapped in a nonproductive folding intermediate that is protease sensitive. Alternatively, exposure of hydrophobic regions could lead to prolonged binding of chaperones and/or be a degradation signal to the ER quality control system (*27*).

To demonstrate in vivo protein folding problems of mutant protein, an unfolded protein response reporter system was constructed. Expression of mutations to the conserved 708-TGDGVNDSPALKK segment of Na,K-ATPase in this reporter strain at 35 and 15 °C showed that the mutant proteins were selectively recognized by the ER quality control system as misfolded or unfolded protein at the higher temperature (Figure 6). This is in accordance with escape of mutant protein from ER (Figure 7) and accumulation at 15 °C (Figures 2 and 3). Temperature-sensitive mutations fall into two groups. Amino acid substitutions either interrupt the protein folding pathway or create proteins that fold to the correct final conformation but are thermodynamically unstable (*32*). From data in Table 2 it is evident that all alanine substitutions had $T_{1/2}$ values for temperature inactivation close to wild type, demonstrating that the folded conformation of mutant protein is as stable as wild type. It is particularly important that no inactivation was observed for mutant protein at 35 °C, the restrictive temperature for protein accumulation. Therefore, mutations to the conserved

708-TGDGVNDSPALKK segment fall into the class of temperature-sensitive synthesis mutants.

It has been shown that α-subunits C-terminally truncated up to M5 are stable when expressed in *Xenopus* oocytes in contrast to full-length Na,K-ATPase (*17*, *20*). Therefore, the first four transmembrane segments and the large cytoplasmatic loop do not expose any degradation signals during biosynthesis. However, inefficient membrane insertion of the C-terminal membrane pairs results in exposure of degradation signals both to the cytoplasm and to the ER lumen (*20*). Since the conserved region examined in this study is connected to M5 through a parallel *β*-sheet (*14*), local folding problems may result in improper membrane insertion of M5 and the following C-terminal membrane segments, leading to exposure of degradation signals. However, since mutations to the conserved segment induce the unfolded protein response, mutant proteins must be recognized from the ER lumen. Therefore, as an alternative, mutations might interfere with helix packing of the C-terminal membrane segments, eventually resulting in unfolded protein response. It has very recently been shown that the unfolded protein response pathway increases expression of genes required for ER-associated protein degradation (*29*−*31*). Lack of accumulation of α_1 protein at 35 °C is therefore most likely due to an increased rate of protein degradation caused by activation of the unfolded protein response pathway. Degradation may take place in the proteasome as has previously been shown for misfolded Na,K-ATPase (*20*) and other transmembrane proteins (*42*).

The presence of α_1 mRNA prior to induction shows that the galactose-regulated promotor is leaky (Figure 4). This is in concordance with the observation that α_1-subunit protein is present prior to induction for wild type as well as N713A and S715A (Figure 2D). This initial pool of N713A and S715A proteins disappears after promotor induction at 35 °C with a half-life of approximately 25 h, similar to that observed for wild type in MDCK cells (*43*). Data therefore fit a model in which [^{3}H]oaubain sites present prior to galactose induction are degraded with a usual rate while no new [^{3}H]oaubain sites accumulate after addition of galactose. The reason for accumulation of [^{3}H]oaubain sites before induction could be that the amount of α_1 protein translated from the small amount of α_1-mRNA present can be handled by the ER folding machinery. Induction of the promotors may cause the amount of mutant protein imported into the ER to exceed the folding capacity and activate the unfolded protein response leading to degradation of mutant protein.

Data in the present paper show that residues in the conserved 708-TGDGVNDSPALKK are important for folding of Na,K-ATPase. In the high-resolution 3D structure of Ca-ATPase this sequence is part of a Rossman fold next to the phosphorylation site (*14*). Alanine scanning mutagenesis showed that the transiently phosphorylated aspartate and conserved neighbor residues in yeast H-ATPase are important for folding and intracellular transport (*10*−*12*). Several of these mutant proteins accumulated in the ER in a trypsin-sensitive conformation indicating misfolding. Interactions between these two conserved segments may be of importance for correct folding and/or membrane insertion of the C-terminal helices in P-type ATPases.

Residues in the conserved segment studied have also been shown to contribute to Mg^{2+} binding in the E_1 conformation of Na,K-ATPase (*15*). The binding of metal ions can be important for protein stability (*44*, *45*). Mutation of metal binding sites has also been shown to reduce expression levels of heterologous protein (*46*). Lack of Mg^{2+} binding cannot be the cause of the observed folding problems and degradation of Na,K-ATPase mutants described in this study since only D710, N713, and possibly D714 are implicated in Mg^{2+} coordination whereas the remaining mutants have wild-type characteristics (*15*). Residues in the conserved 708-TGDGVNDSPALKK segment may therefore contribute side chains of importance for Mg^{2+}, P_i, or VO_4 interactions (*15*) and in addition be important for correct overall folding of P-type ATPases.

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