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Genetic characterization of the ^{534}DPPR motif of the yeast plasma membrane H$^+$-ATPase

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Abstract

The highly conserved motif ^{534}DPPR of *Saccharomyces cerevisiae* H$^+$-ATPase, located in the putative ATP binding site, has been mutagenized and the resulting 23 mutant genes conditionally expressed in secretory vesicles. Fourteen mutant ATPases (D534A, D534V, D534L, D534N, D534G, D534T, P535A, P535V, P535L, P535G, P535T, P535E, P535K and R537T) failed to reach the secretory vesicles. Of these mutants, nine (D534N, D534T, P535A, P535V, P535L, P535G, P535T, P535E and P535K) were not detected in total cellular membranes, and five (D534A, D534V, D534G, D534L and R537T) were retained at the endoplasmic reticulum and exhibited a dominant lethal phenotype. The remaining mutants (D534E, R537A, R537V, R537L, R537N, R537G, R537E, R537K and R537H) reached the secretory vesicles at levels similar to that of the wild type. Of these, six (R537A, R537V, R537L, R537N, R537G, and R537E) showed severely decreased ATPase activity compared to the wild type enzyme, and three (D534E, R537K and R537H) rendered an enzyme with an altered K_m for ATP.

Keywords: H$^+$-ATPase; Plasma membrane; Site-directed mutagenesis; *Saccharomyces cerevisiae*

1. Introduction

The yeast plasma membrane H$^+$-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump essential for nutrient uptake and intracellular pH regulation [1,2]. The ATPase belongs to the P-type ion translocating ATPase family, which forms an acyl-phosphate intermediate during catalysis and is sensitive to vanadate [3].

The genes encoding members of the P-type ATPase family from bacterial, fungal, plant and animal cells have been cloned and sequenced. Hydropathy analysis has revealed a common topology for the P-ATPases [4,5], with two hydrophilic domains and 10 transmembrane segments. This model is in agreement with recent data from cryoelectron microscopy of frozen–hydrated crystals of sarcoplasmic reticulum Ca^{2+}-ATPase and *Neurospora crassa* H$^+$-ATPase [6,7]. Comparison of P-ATPase amino acid sequences showed that they share several highly conserved motifs, including consensus sequences for ATP binding and phosphorylation [8–12]. In yeast H$^+$-ATPase, sequence analysis suggests that the putative nucleotide binding site is located in the major hydrophilic domain of the enzyme at positions 430–

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680 [13]. Moreover, results based on chemical labeling experiments of the major hydrophilic domain of the sarcoplasmic reticulum Ca^{2+}-ATPase and yeast H^+-ATPase suggest the involvement of this region of the enzyme in ATP binding [14,15].

In previous studies, we isolated a mutations at Pro-536 of the yeast H^+-ATPase that acts as an allele-nonspecific suppressor, since it was able to suppress mutations located in different regions of the enzyme [16,17]. Genetic characterization of site-directed mutants at this residue suggest that Pro-536 is an important determinant in enzyme biogenesis [18]. Pro-536 is located within the sequence motif ^{534}DPPR, which is a likely loop in the structure of the enzyme and is believed to form part of the ATP binding site [13]. This finding prompted us to further characterize the effect of ^{534}DPPR substitutions on enzyme function. In this report, we show that mutation at Asp-534 and Arg-537 alter the apparent K_m of the enzyme for ATP, and that Pro-535 is important for the biosynthesis and/or stability of the protein.

2. Materials and methods

2.1. Yeast strains and growth media

Saccharomyces cerevisiae strain SY4 is GAL^+ *MAT*a *ura3 leu2 his4 sec6 pma1*::*YIpGAL-PMA1* and was used to express the ATPase in secretory vesicles [19]. Strain XZ611 is Gal^+ *MAT*a *ura3 leu2 trp1* [20] and was used in immunofluorescence experiments. Synthetic media with 2% dextrose (SD), 2% raffinose (SR) or 2% galactose (SG) and the appropriate requirements were used [21]. Yeast cells were transformed using the lithium acetate procedure [22]. To test the dominant lethality of the *pma1* alleles, transformants were transferred to SR medium and, after growth, suspended in water at a cell density of 2×10^7 cell/ml, and 5 μl was dropped on SG.

2.2. Site-directed mutagenesis

A 2.2-kb *Xba*I fragment of PMA1 subcloned into M13mp19 [25] was subjected to oligonucleotide-directed mutagenesis using the method of Taylor et al. [26] with the Amersham mutagenesis system (Amersham Corp.). All ATPase fragments subjected to mutagenesis were sequenced by the dideoxy method [27]. Mutagenesis efficiency ranged from 70 to 100%, and the only sequence changes observed were those introduced by the mutagenic oligonucleotides. The ATPase gene fragments containing the mutations were liberated from the M13mp19 replicative form and inserted into a 5.0-kb *Hin*dIII fragment containing the ATPase gene [23] inserted into the pSB32 centromeric plasmid [24] in which the 2.2-kb *Xba*I fragment had previously been deleted.

2.3. Construction of hemagglutinin (HA)-tagged pma1 alleles

The HA-tagged *pma1* alleles used in this study were generated by exchange of a 3.4-kb *Bst*EII–*Hin*dIII fragment containing the desired mutation, with the corresponding fragment in plasmid pFP302. The plasmid pFP302 is a derivative of the *URA3* single copy plasmid YCp50 [24] and it carries a HA-tagged wild type *PMA1* under the control of the *GAL1* promoter [28].

2.4. Expression of ATPase in secretory vesicles

The 2.6-kb *Bam*HI–*Hin*dIII fragments of *PMA1* containing the substitutions were ligated into plasmid pPMA1.2 [19] that had been digested with *Bam*HI and *Hin*dIII. After the fragments were moved into plasmid pPMA1.2, a 3.77-kb *Sac*I–*Hin*dIII fragment containing the entire ATPase coding sequence was cloned into vector YCp2HSE [19], placing the mutant alleles under the control of a heat shock-inducible promoter. The resulting plasmids were transformed into strain SY4 [19]. This strain has the chromosomal *PMA1* gene under the control of the *GAL1* promoter and also carries the *sec6-4* mutation that blocks fusion of secretory vesicles with the plasma membrane. For expression studies, transformed SY4 cells were grown to mid-exponential phase ($OD_{660}\approx0.5$) on SG and shifted to SD for 3 h to turn off transcription of chromosomal *PMA1*, then shifted to 37°C for 2 h to turn on expression of the plasmid-borne gene and block fusion of secretory vesicles with plasma membrane. Secretory vesicles were then isolated by differential and sucrose gradient centrifugation as described [29].

2.5. Fluorescence microscopy

Strain XZ611, transformed with different *pma1* alleles, was selected in SD medium. To induce *GAL1*::*pma1* gene expression, cells were cultured in SR medium overnight at 30°C, collected, resuspended in SG medium and cultured for 5 h at 30°C. Cells were fixed and stained for immunofluorescence as described [30]. Localization of HA-tagged Pma1 was determined by immunofluorescence using a rhodamine-conjugated anti-HA antibody (Boehringer-Mannheim, Mannheim, Germany).

2.6. Biochemical methods

Immunoquantification of the ATPase was performed as described [31] using affinity-purified rabbit polyclonal antibody against yeast ATPase [32]. ATPase activity was assayed at pH 6.5 with 5 mM ATP [33]. H^+ pumping into secretory vesicles was monitored by fluorescence quenching of acridine orange [19]. For the determination of K_m values, the ATP concentration was varied from 0.2 to 6 mM and the concentration of free Mg^{2+} was 1 mM. To determine K_i values, the concentration of orthovanadate and erythromycin B was varied from 0.5 to 20 μM. Protein concentration was determined by the Bradford method [34] with the Bio-Rad Protein Assay Reagent and bovine IgG as standard. Total membrane proteins were separated by 8% SDS–PAGE using the Laemmli system [35]. Western blot with second antibody conjugated to alkaline phosphatase (Bio-Rad) was as described [36].

3. Results

The aim of this study was to explore the structure–function relationships in the region surrounding Pro-536. Amino acids subjected to site-directed mutagenesis were selected after a comparative study of the sequence from 22 representative P-type ATPases differing in cation specificity and evolutionary origin. There is a motif of high similarity from Asp-534 through Arg-537; upstream of Asp-534 and downstream of Arg-537 the degree of conservation decreases (Fig. 1). Within this motif Asp-534 is invariant and Arg-537 is nearly invariant, Lys replaces the latter in heavy metal pumps. Pro-535 is conserved among H^+-, Na^+-, Na^+K^+-, H^+K^+-, Ca^{2+}- and Mg^{2+}-ATPases; it is replaced by different amino acids (Ser, Glu, Val, Thr, Ala, and Ile) in heavy metal pumps and *Escherichia coli* K^+-ATPase. Based on the alignment of Fig. 1, we carried out site-directed mutagenesis of Asp-534, Pro-535 and Arg-537. Each of the above-mentioned amino acids was replaced with residues of different size and charge (Table 1).

Organisms	Locus	Specificity	Sequences
S. cerevisiae	*PMA1*	H^+	532 CMDPPRDDT
A. thaliana	*AHA1*	H^+	486 LFDPPRHDS
D. acidophila	*DHA1*	H^+	512 MFDPPRHDT
S. cerevisiae	*ENA1*	Na^+	647 IYDPPRMET
Human	*ATP1A1*	Na^+/ K^+	591 MIDPPRAAV
Human	*ATP4A*	H^+/ K^+	602 MIDPPRAYV
S. cerevisiae	*PMR1*	Ca^{2+}	598 MNDPPRPNV
K. lactis	*KlPMR1*	Ca^{2+}	582 MPDPPRPIV
L. esculentum	*LCA1*	Ca^{2+}	622 LRDPPREEV
Rabbit	*SRCA*	Ca^{2+}	588 MLDPPRIEV
S. cerevisiae	*PMC1*	Ca^{2+}	736 IQDPLRAGV
Human	*ATP2B2*	Ca^{2+}	682 IEDPVRPEV
A. thaliana	*ACA2*	Ca^{2+}	657 IKDPVRPGV
S. cerevisiae	*PCA1*	Cu^{2+}	1022 LEDSLRADA
S. cerevisiae	*CCC2*	Cu^{2+}	761 INDEVKHDS
E. hirae	*CopB*	Cu^{2+}	583 LGDVIKPEA
Human	*ATP7A*	Cu^{2+}	1270 IADIVKPEA
Human	*ATP7B*	Cu^{2+}	1234 IADAVKQEA
S. aureus	*TnpA*	Cd^{2+}	643 VADEVREIS
L. lactis	*CadA*	Cd^{2+}	526 VADEVRDSS
S. typhimurium	*MgtA*	Mg^{2+}	565 FLDPPKEIT
E. coli	*KdpA*	K^+	445 LKDIVKGGI

Fig. 1. Alignment of the ^{534}DPPR region in P-ATPases. Amino acid sequences were aligned using the Pileup program of the University of Wisconsin Genetic Computer Group. The enzymes were grouped depending on cation specificity. The EMBL database accession numbers from top to bottom are: P05030, P20649, P54210, P13587, P05023, P20648, P13586, O74296, Q42883, P20647, P38929, P20020, O81108, P38360, P38995, P05425, Q04656, P35670, Q53649, U78967, P36640 and P03960.

3.1. Expression of mutant ATPases in secretory vesicles

Because some mutations could be lethal [20,28,37,38], a transient expression system [19] was used to characterize the effect of the mutation on

Table 1
Analysis of mutant ATPases accumulated in secretory vesicles

Allele expressed	Amount of ATPase[a]	ATPase activity[b]		H^+ transport[c]	
		Uncorrected (μmol/min/mg)	Corrected (%)	Uncorrected (%*Q*/mg)	Corrected (%)
Wild type	100	1.20	100	725	100
None[d]	5	0.03	n.d.[e]	n.d.	n.d.
D534A	5	0.04	n.d.	n.d.	n.d.
D534V	8	0.03	n.d.	n.d.	n.d.
D534L	7	0.02	n.d.	n.d.	n.d.
D534N	5	0.03	n.d.	n.d.	n.d.
D534G	8	0.05	n.d.	n.d.	n.d.
D534T	5	0.05	n.d.	n.d.	n.d.
D534E	100	0.64	53	335	46
P535A	6	0.06	n.d.	n.d.	n.d.
P535V	6	0.05	n.d.	n.d.	n.d.
P535L	7	0.03	n.d.	n.d.	n.d.
P535G	5	0.02	n.d.	n.d.	n.d.
P535T	3	0.04	n.d.	n.d.	n.d.
P535E	8	0.02	n.d.	n.d.	n.d.
P535K	9	0.01	n.d.	n.d.	n.d.
R537A	95	0.20	17	b.s.[f]	b.s.
R537V	90	0.24	22	b.s.	b.s.
R537L	98	0.28	23	b.s.	b.s.
R537N	80	0.16	16	b.s.	b.s.
R537G	92	0.23	20	b.s.	b.s.
R537T	7	0.04	n.d.	n.d.	n.d.
R537E	75	0.16	10	b.s.	b.s.
R537K	80	0.58	60	320	55
R537H	105	0.60	47	330	43

[a]Quantified by immunoassay. The value for the wild type was considered 100%.
[b]ATPase activity was assayed at pH 6.5 with 5 mM ATP. The specific activity of each secretory vesicle preparation was measured as the difference between hydrolysis in the absence and presence of 250 μM orthovanadate. Values are the average of two independent experiments differing less than 15%. Each mutant value was corrected for the relative amount of mutant protein in the secretory vesicle preparation.
[c]Fluorescence quenching of acridine orange was used to monitor pumping of protons into secretory vesicles. Similar values (within 15%) were obtained with two different secretory vesicle preparations independently isolated. Each mutant value was corrected for the relative amount of mutant protein in the secretory vesicle preparation.
[d]Secretory vesicles were isolated from SY4 transformed with the vector YCp2HSE carrying no *PMA1* gene.
[e]n.d.: not determined.
[f]b.s.: ATP-dependent fluorescent quenching was below the limits of sensitivity of the assay ($<20\%$ of the wild type rate).

enzyme function. The mutant genes were placed under the control of a heat shock-inducible promoter in the plasmid YCp2HSE [19]. Plasmid YCp2HSE containing the mutant genes was transformed into strain SY4 [19]. This strain has the chromosomal *PMA1* gene under the control of the *GAL* promoter and also carries the temperature-sensitive *sec6-4* mutation that blocks fusion of secretory vesicles with the plasma membrane. Thus, when cells were incubated in galactose medium at 23°C, only the chromosomal wild type ATPase was produced, and when cells were transferred to glucose medium at 37°C, wild type ATPase expression was repressed and mutant ATPase was expressed. Since strain SY4 carries a temperature-sensitive sec6-4 allele, the shift to 37°C also led to accumulation of the newly synthesized mutant ATPase in secretory vesicles. Secretory vesicles were purified by differential and sucrose gradient centrifugation [29] for analysis of the mutant ATPases; Table 1 summarizes the behavior of the

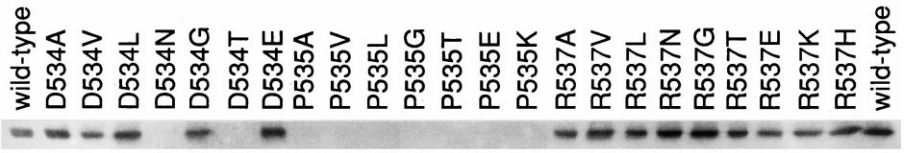

Fig. 2. Accumulation of wild type and mutant ATPases in total cellular membrane. Strains are recombinants carrying either the PMA1 or mutant genes under the control of a heat shock-inducible promoter into plasmid YCp2HSE. Cells were grown in SG medium and transferred to SD to turn off expression of the chromosomal *PMA1* gene, and then shifted to 38°C for 60 min to induce expression of the *pma1* alleles. Total plasma membrane was analyzed by immunoblot using affinity-purified rabbit polyclonal antibody against yeast ATPase.

mutant enzymes. The expression levels of D534E, R537A, R537V, R537L, R537N, R537G, R537E, R537K and R537H were similar to the wild type control. All remaining mutant enzymes (D534A, D534V, D534L, D534N, D534G, D534T, P535A, P535V, P535L, P535G, P535T, P535E, P535K and R537T) failed to reach the secretory vesicles. In a parallel experiment, total cell membranes were isolated and analyzed by Western blot (Fig. 2). In many cases, the mutant protein could be detected at near-normal levels 60 min after the temperature shift, but for nine mutants (D534N, D534T, P535A, P535V, P535L, P535G, P535T, P535E and P535K) no Pma1 protein could be detected in total cellular membranes. In an alternative experiment cells carrying HA-tagged versions of these mutant ATPase genes under the control of the GAL1 promoter were shifted from glucose- to galactose-containing medium. Total cellular membranes were purified at different times of induction (30 min, 1, 2, 3, 4 and 5 h) and analyzed by Western blot using anti-HA antibody. None of the above-mentioned mutant proteins could be detected (data not shown). These results suggest that these mutations could affect the synthesis and/or the short-term stability of the enzyme; these mutants were not studied further. Secretory vesicle preparations were next assayed for their ability to hydrolyze ATP and analyzed for ATP-dependent proton transport (Table 1). Three mutants (D534E, R537K and R537H) had hydrolysis and transport activities ranging from 47 to 60% and from 43 to 55% when correction for the expression level in the secretory vesicle preparation is considered. Six mutants showed significant reduction in activity (R537A to 17%, R537V to 22%, R537L to 23%, R537N to 16%, R537G to 20% and R537E to 10%) and accordingly the proton pumping activities were too low ($<20\%$ of wild type) to give a measurable signal in the quenching assay.

We next analyzed the kinetic properties of the D534E, R537K and R537H mutant enzymes (Table 2). The apparent K_M for ATP of the mutants differed only slightly of the wild type enzyme. Nevertheless, the mutant enzymes were more resistant to erythromycin B inhibition. Erythromycin B inhibits the ATP hydrolysis catalyzed by the ATPase by binding to the enzyme nucleotide binding site [39]. These results thus suggest that mutations D534E, R537K and R537H could disturb the ATP binding domain structure.

3.2. Subcellular location of the mutant ATPases

Because D534A, D534V, D534L, D534G and R537T mutations prevent accumulation of Pma1p into secretory vesicles although they accumulate in

Table 2
Kinetic characterization of Asp-534 and Arg-537 mutants

Mutation	K_M(ATP) (mM)	K_i(vanadate) (μM)	K_i(erythromycin B) (μM)	pH optimum
Wild type	1.6	2.0	1.0	6.00
D534E	3.3	1.8	5.0	6.00
R537K	4.0	2.3	5.0	6.00
R537H	3.0	2.0	2.5	6.00

Values were determined as described in Section 2 and are the average of two independent experiments.

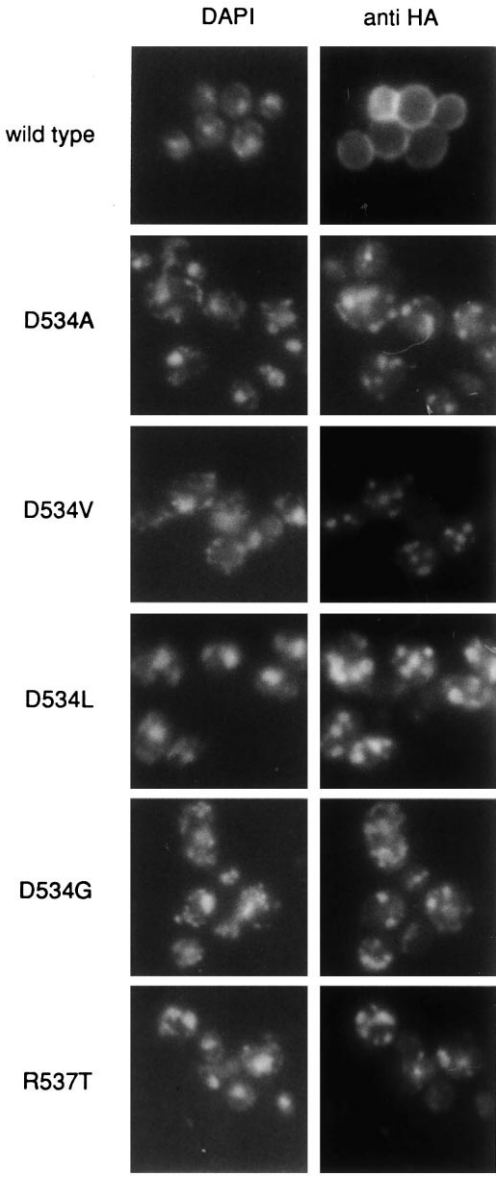

Fig. 3. Accumulation of the HA-tagged mutant protein in cytoplasmic structures. The transformants carried either the HA-tagged *PMA1* or HA-tagged *pma1* genes under the control of the *GAL1* promoter. After galactose induction, cells were stained for the nucleus (DAPI) or immunodecorated for the HA-tagged Pma1p using a rhodamine-conjugated anti-HA antibody (anti HA).

total cellular membranes, we next studied the location of the mutant ATPases. The HA-tagged genes were placed under the control of the *GAL1* promoter in a centromeric plasmid and used to transform a wild type strain (XZ611). The location of the mutant HA-tagged proteins was studied in galactose-cultured cells by immunofluorescent staining of mutant Pma1p with the anti-HA monoclonal antibody. In D534E, R537A, R537V, R537L, R537N, R537G, R537E, R537K and R537H, the anti-HA antibody decorated the cell periphery (not shown); this was also the case for the wild type control (Fig. 3). In contrast, in D534A, D534V, D534L, D534G and R537T, the HA-tagged mutant ATPase appeared to accumulate in cytoplasmic structures. Such prolifer-

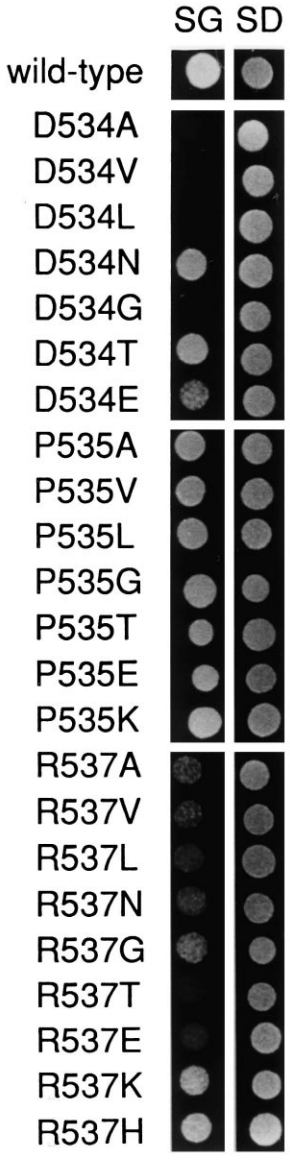

Fig. 4. Drop test for growth of yeast strains expressing mutant alleles of PMA1. Strains are recombinants, carrying the *PMA1* wild type allele or the indicated *pma1* gene under the control of the galactose-inducible promoter. The test was performed as indicated in Section 2. Plates were photographed after 36 h at 30°C.

ation of intracellular structures has been reported in cells expressing dominant lethal Pma1 proteins [20,28,38]. It was therefore of interest to determine whether D534A, D534V, D534L, D534G and R537T have, in fact, a dominant lethal phenotype. To this end, cells expressing the HA-tagged mutant ATPase genes under the control of the GAL1 promoter were plated on galactose medium (Fig. 4). As predicted, D534A, D534V, D534L, D534G and R537T failed to grow on galactose medium, suggesting that they are dominant lethal alleles. It is interesting to note that R537A, R537V, R537L, R537N, R537G, and R537E mutants, although not retained at the endoplasmic reticulum and slightly active, exerted an inhibitory effect on cell growth, suggesting that they interfere with the function of the wild type ATPase. Further work is necessary to determine the specific mechanism affected by these Arg-537 mutations.

4. Discussion

In this study, the role of amino acids Asp-534, Pro-535 and Arg-537, predicted to be located in the ATP binding domain of the yeast plasma membrane H^+-ATPase, has been analyzed by site-directed mutagenesis.

Replacement of Asp-534 by either nonpolar or small polar residues resulted in enzymes severely impaired in their biogenesis; only the conservative change Asp→Glu at this position resulted in an active enzyme. Mutants at Arg-537 were close to normal in biogenesis in almost all cases, and had specific activities that ranged from 10% (R537E) to 60% (R537K). Analysis of the kinetic properties of the D534E, R537K and R537H mutant enzymes showed that the apparent K_ms of these enzymes were slightly altered and that mutant enzymes were resistant to the ATP binding inhibitor erythromycin B. Although these data do not lead to definitive conclusions, it appears that the phenotype conferred by the D534E, R537K and R537H mutations could be explained if we assume that these mutations disturb the nucleotide binding site. The Asp-534 and Arg-537 residues are equivalent to the amino acids of the sarcoplasmic reticulum Ca^{2+}-ATPase Asp-601 and Arg-604, respectively, which have been shown to be involved in nucleotide binding [40]. Further studies involving chemical labeling experiments of the mutant ATP binding domains overexpressed in *E. coli* together with structural work (nuclear magnetic resonance, X-ray crystallography) will definitively clarify the role of the ^{534}DPPR motif.

With respect to Pro-535, the fact that P535A, P535V, P535L, P535G, P535T P535E or P535K were not detected in total cellular membrane suggests that this residue is important for the biosynthesis and/or stability of the ATPase. The present data do not allow us to determine whether these mutations affect the biosynthesis or the stability of the enzyme, but comparison with published data for other *PMA1* mutants, which exhibit the same phenotype [41], indicates that it may be reasonable to consider that Pro-535 mutations affect the enzyme stability. In the case of the sarcoplasmic reticulum Ca^{2+}-ATPase, replacement of this residue (Pro-602) by leucine has no noticeable effect on ATPase biogenesis and activity [40]. Despite sequence conservation, mutations at Pro-535 of the yeast H^+-ATPase appear to have a much more deleterious effect on the enzyme than do equivalent changes in Ca^{2+}-ATPase. This dissimilar behavior between PMA1 mutations and their counterparts in other yeast and mammalian P-ATPases has been observed with mutations at the phosphorylation site [38]. Further data must be obtained to understand the molecular basis for this striking difference.

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