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Functioning of *Saccharomyces cerevisiae* Pma1 H^+-ATPase Carrying the Minimal Number of Cysteine Residues

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Abstract—Pma1 H^+-ATPase is the primary proton pump in the plasma membrane of the yeast *Saccharomyces cerevisiae*. It generates an electrochemical proton gradient, thus providing energy for secondary solute transport systems. The enzyme contains nine cysteines, three (Cys148, Cys312, and Cys867) in transmembrane segments and the rest (Cys221, Cys376, Cys409, Cys472, Cys532, and Cys569) in the cytosolic parts of the molecule. Although individually they are not essential for the functioning of the ATPase, substitution of all of them leads to the loss of enzyme activity and impairment of biogenesis. By means of site-directed mutagenesis combined with other molecular-biological and biochemical methods, this work defines different combinations of minimal cysteine content that are consistent with ATPase function.

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Cation-transporting P2-ATPases belong to a family of P-ATPases that are widespread in different organisms [1]. They include Mg^{2+}-ATPases of prokaryotes, eukaryotic H^+- and Ca^{2+}/Mn^{2+}-ATPases of fungi and yeasts, H^+-ATPases of plants, and Na^+/K^+-, H^+/K^+-, and Ca^{2+}-ATPases of mammalian cells [1, 2]. Since 1986, when the first P2-ATPase sequences of *Saccharomyces cerevisiae* [3] and *Neurospora crassa* [4] were published, several dozens of genes encoding these enzymes have been cloned. Despite their significant physiological diversity, they all have similar topology and structure. This fact has been confirmed recently by the study of crystal structure of the Ca^{2+}- [5-8], Na^+/K^+- [9], and H^+-ATPases [10, 11] of mammals [5-9], plants [11], and fungi [10]. Nevertheless, the mechanisms of functioning of these physiologically important cation pumps have not yet been fully elucidated.

Pma1 H^+-ATPase is a vital enzyme acting as a primary proton pump; disruption of the *PMA1* gene encoding this enzyme is lethal for the cell [3]. Like other P2-ATPases, the *S. cerevisiae* plasma membrane Pma1 H^+-ATPase is an integral membrane protein with 10 transmembrane segments [5-11]. Its catalytic part faces the cytosol, where ATP binding is accompanied by formation of an essential β-aspartyl phosphate reaction intermediate. The energy generated by hydrolysis of this intermediate is used to pump protons out of the cell through the proton-binding site formed by several amino acid residues located in transmembrane segments, with M4, M5, M6, and M8 being most essential. As a result, transmembrane electrochemical gradient of protons ($\Delta\mu_{H^+}$) is generated, thus providing a source of the energy for secondary solute transport systems and playing an important role in maintenance of ion homeostasis in the yeast cell. During the reaction cycle, the enzyme undergoes significant conformational changes similar to Ca^{2+}-ATPase [7].

The *S. cerevisiae* Pma1 enzyme contains only nine cysteine residues, unlike its mammalian counterparts with 15-33 cysteines [12]; it allows more effective estimation of their role in enzyme functioning by means of molecular biological, genetic, and biochemical approaches. Site-directed mutagenesis of Cys residues is one of the approaches to study the structure–function relationship in the Pma1 ATPase including the measurement of distances between different parts of the enzyme during the reaction cycle. On one hand, substitution of Cys residues provides better understanding of their role in biogenesis and structure–function relationship in ATPase; on the other hand, it is possible to use mutant enzymes, in particular those with multiple Cys replacements, for selective labeling of different parts of ATPase with radioactive, fluorescent, or spin-labeled SH reagents

to study the enzyme topology, structure, and conformational changes during the reaction cycle. Mutant forms with minimal cysteine content and maintenance of functional activity of the enzyme provide additional advantages, since new Cys could be introduced at points of interest.

We previously substituted all Cys residues in the *S. cerevisiae* Pma1 ATPase, one at a time, with Ala or Ser [13]. None of them appeared to be vital for expression and functioning of the enzyme, but simultaneous replacement of them all resulted in the loss of ATPase activity with substantial impairment of its biogenesis [13]. Two strains with minimal Cys content were constructed: a *one-cysteine* strain containing only Cys409 and a *two-cysteine* strain having Cys409 and Cys472. However, the expression and activity of these forms were significantly lower than the wild type [13].

The goal of this work was to construct and characterize mutant forms of *S. cerevisiae* Pma1 H^+-ATPase carrying minimal content of Cys residues keeping the enzyme functionally active. In this study, we have constructed and partially characterized new mutants, including *one-*, *two-*, and *three-cysteine* forms; three of the latter with different combinations of Cys148, Cys312, Cys376, Cys409, and Cys472 proved to be the most promising for further studies.

MATERIALS AND METHODS

Isolation of secretory vesicles. The yeast *S. cerevisiae* strain SY4 (*MATa; ura3-52; leu2-3, 112; his4-619; sec6-4; GAL; pma1::YIpGAL-PMA1*) with chromosomal and plasmid copies of the *PMA1* gene was used throughout the work [13, 14]; centromeric plasmid YCp2HSE was introduced into a cell by transformation as described [13, 14]. The chromosomal copy of the wild type ATPase gene was placed under the control of *GAL1* promoter (P_{GAL}-*PMA1*) and the plasmid allele was under heat shock-inducible *HSE* promoter (P_{HSE}-*pma1*) [13, 14]. The plasmid-carried *pma1* gene encoded either the wild type or mutant enzyme [13]. Strain SY4 also carries a temperature-sensitive mutation in the *SEC6* gene, which blocks the fusion of secretory vesicles to the plasma membrane under heat shock and leads to accumulation of secretory vesicles [15].

The yeast was grown on a medium with 2% galactose at 23°C [13, 14]. In the mid-exponential phase, cells were washed free from galactose and transferred to medium containing 2% glucose, followed by a heat shock after 3 h (37°C for 2 h). Ten minutes before the heat shock termination, 10 mM NaN_3 was added to the cells; then the cells were chilled on ice for 10 min, sedimented, and washed twice with 10 mM NaN_3. To obtain spheroplasts, the cells were resuspended in 1.4 M sorbitol, 10 mM NaN_3, 1 mM EDTA, 50 mM K_2HPO_4, pH 7.5, 50 mM 2-mercaptoethanol, and incubated with Zymolyase (ICN, USA) for 30 min at 37°C. All subsequent procedures were carried out at 0-4°C. Spheroplasts were treated with concanavalin A to make plasma membranes heavier in order to remove them during low-speed centrifugation. Then the spheroplasts were sedimented and resuspended in 0.4 M sorbitol, 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 7.2, and protease inhibitors (1 mM diisopropyl fluorophosphate, 2 μg/ml chymostatin, and leupeptin, pepstatin, and aprotinin, 1 μg/ml each) [13]. The inhibitors were present at all subsequent steps. The spheroplasts were lysed in a Potter homogenizer and secretory vesicles were isolated by differential centrifugation and gel filtration on a column with Sephacryl S-1000 (Pharmacia, Sweden) [13, 14]. The secretory vesicles were resuspended in 0.8 M sorbitol, 10 mM triethanolamine-acetic acid, pH 7.2, containing all of the protease inhibitors except for diisopropyl fluorophosphate [13].

Site-directed mutagenesis. The Amersham kit (Amersham Corp., USA) for oligonucleotide-directed mutagenesis was employed to introduce single point mutations using cassette plasmids – modified versions of Bluescript (Stratagene, USA) carrying fragments of the *PMA1* gene (*Cla*I-*Bst*EII; *Bst*EII-*Eco*RI; *Sty*I-*Bam*HI; *Bam*HI-*Sac*I) [13, 14]. To obtain multiple Cys substitutions, oligonucleotides carrying point Cys-to-Ala (or Cys-to-Ser) mutations complementary to the corresponding fragments of these plasmids were synthesized, and mutated versions of these plasmids were constructed. Then the corresponding fragments of plasmid pPMA1.2 carrying the entire coding sequence of the gene were replaced by restriction endonucleases BamHI, BglII, BstEII, ClaI, EcoRI, HindIII, SacI, SalI, StyI, and T4 DNA ligase (NEB, USA) with the same fragments carrying Cys replacements [13]. Each fragment was sequenced to verify the presence of mutations and the absence of unwanted base changes. The complete sequence of the gene (*Hind*III-*Sac*I) was then transferred from pPMA1.2 into plasmid YCp2HSE used to transform the SY4 cells [13, 14].

Quantitation of expressed ATPase. The amount of ATPase in secretory vesicles was estimated by immunoblotting. To prevent proteolysis, total protein of the vesicles was precipitated with 10% trichloroacetic acid and then subjected to SDS-gel electrophoresis, transferred to Immobilon-P membranes (Millipore Corporation, USA), and treated with polyclonal antibodies against the closely related Pma1 H^+-ATPase of *N. crassa* [4] followed by incubation with ^{125}I-labeled protein A (ICN) as described [13]. The amount of ^{125}I-labeled protein A bound to the ATPase–antibody complex was calculated by means of a Phosphorimager equipped with ImageQuant software (Molecular Dynamics, USA) and then used to quantify the enzyme amount in the samples [13]. The expression level of mutant Pma1 ATPase in

secretory vesicles was calculated relative to the wild-type control run in parallel.

ATP hydrolysis. ATP hydrolysis was carried out at 30°C in 0.5 ml of incubation mix containing 5 mM $MgSO_4$, 5 mM Na_2ATP, 50 mM MES-Tris, pH 5.7, 5 mM KN_3, and an ATP-regenerating system (5 mM phosphoenolpyruvate and 50 μg/ml pyruvate kinase) in the absence and presence of 100 μM sodium orthovanadate [13].

ATP-dependent H^+-transport. The ΔpH generated by the ATPase was monitored at 29°C by fluorescence quenching of pH-sensitive dye acridine orange using a Hitachi (Japan) F2000 spectrofluorimeter (excitation, 430 nm; emission, 530 nm) [13, 14]. Secretory vesicles (50 μg protein) were suspended in 1.5 ml of 0.6 M sorbitol, 100 mM KCl, 5 mM Na_2ATP, 2 μM acridine orange, 20 mM HEPES-NaOH, pH 6.7; after stabilization of baseline fluorescence (70-100 sec), the reaction was started by adding 10 mM $MgCl_2$ [13].

Protein assay. Protein was determined by a modified Lowry method [16] with bovine serum albumin used as standard.

RESULTS AND DISCUSSION

Topology and conservation of Cys residues. Of the nine Cys residues (in positions 148, 221, 312, 376, 409, 472, 532, 569, 867) in the *S. cerevisiae* plasma membrane Pma1 H^+-ATPase, three (Cys148, Cys312, and Cys867) reside in transmembrane segments M1, M3, and M10, respectively, and the rest in the cytosolic part of the enzyme; all of them, except for Cys221, are located in the central catalytic domain (Table 1 and Fig. 1). Membrane Cys residues identical to Cys148 and Cys867 are present in almost all Pma H^+-ATPases of ascomycetes; only in single cases they are replaced by Ile or Thr (Table 2). Cys312 is present in less than half of the ascomycetous Pma H^+-ATPases; its substitutions are more variable: Ser, Thr, Ala, and Gly (Table 2). Among the cytosolic residues, Cys376 and Cys472 are most conservative. Cys376 is a part of conservative motif CSDKTGT, which contains Asp378 forming the reaction intermediate; it is present not only in all Pma but also in all P2-ATPases [13]. Residues Cys221, Cys409, Cys532, and Cys569 are present in most Pma H^+-ATPases of ascomycetous fungi (Table 2). Since membrane residues Cys148 and Cys867 and especially cytosolic Cys376 and Cys472 are most conservative (Table 2), one may expect them to be most important for structure–function relationship of the enzyme.

Single Cys substitutions. To study the role of Cys residues, we used the previously developed method of secretory vesicle isolation [14] with some modifications [13]. The wild-type or mutated *pma1* gene carrying substitutions of Cys residues and controlled by *HSE* promoter was subcloned into expression centromeric plasmid YCp2HSE, which then was used to transform *S. cerevisiae* SY4 strain, where the chromosomal parental *PMA1* gene was under the control of *GAL1* promoter [13, 14].

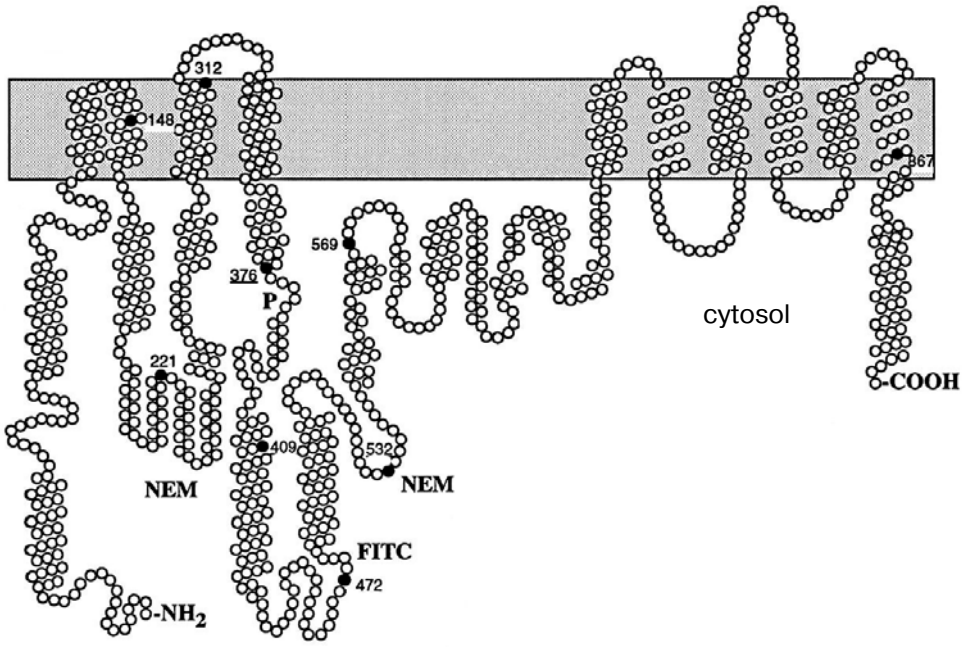

Fig. 1. Topology of the yeast plasma membrane H^+-ATPase. Positions of cysteines are marked by dark circles and numbers. Abbreviations: P, phosphorylation site (Asp378); FITC, fluorescein-5′-isothiocyanate-reactive Lys474; NEM, *N*-ethylmaleimide-binding Cys221 and Cys532. Adapted from [13, 18].

Table 1. Construction of the *S. cerevisiae* Pma1 H^+-ATPase mutants with multiple Cys substitutions

Mutants	Cysteine residue replaced								
	148	221	312	376	409	472	532	569	867
	Cysteine residue localization								
	M1	CD1	M3	CD2	CD2	CD2	CD2	CD2	M10
With several substitutions									
C221,409,532A	–	A	–	–	A	–	A	–	–
C376,409,472A	–	–	–	A	A	A	–	–	–
C376,409,472,532A	–	–	–	A	A	A	A	–	–
C376,472,532A/C409S	–	–	–	A	S	A	A	–	–
C376,532A/C409,472S	–	–	–	A	S	S	A	–	–
C221/376/409/472	A	–	A	–	–	–	A	A	A
C376/409/472/532	A	A	A	–	–	–	–	A	A
C221/376/409/472/532	A	–	A	–	–	–	–	A	A
Three-cysteine									
C148/409/472	–	A	A	A	–	–	A	A	A
C221/409/532	A	–	A	A	–	A	–	A	A
C312/409/472	A	A	–	A	–	–	A	A	A
C376/409/472	A	A	A	–	–	–	A	A	A
C409/569/867	A	A	A	A	–	A	A	–	–
Two-cysteine									
C148/409	–	A	A	A	–	A	A	A	A
C221/409	A	–	A	A	–	A	A	A	A
C312/409	A	A	–	A	–	A	A	A	A
C409/472	A	A	A	A	–	–	A	A	A
C409/532	A	A	A	A	–	A	–	A	A
C312/472	A	A	–	A	S	–	A	A	A
C376/472	A	A	A	–	S	–	A	A	A
One-cysteine									
C409	A	A	A	A	–	A	A	A	A
C472	A	A	A	A	S	–	A	A	A

Note: M1, M3, and M10 are transmembrane segments 1, 3, and 10; CD1 and CD2 are small (1) and large (2) loops of the central catalytic domain; C, cysteine; A, alanine; S, serine.

The yeast was grown at 23°C in medium containing 2% galactose [13, 14]; under these conditions, the wild-type enzyme was synthesized from the chromosomal gene *PMA1* (Fig. 2). When the cells were shifted to glucose-containing medium, the synthesis from the chromosomal gene was interrupted; when the temperature was elevated to 37°C, the Pma1 synthesis started from the plasmid *pma1* gene (encoding either the wild-type ATPase serving as positive control or a mutant one). Strain SY4 also carried temperature-sensitive mutation *sec6-4*, which blocked the fusion of secretory vesicles to the plasma membrane [15]. Therefore, the heat shock resulted in accumulation of secretory vesicles with the enzyme that was newly synthesized from the plasmid *pma1* gene (Fig. 2a). Secretory vesicles isolated from the strain, which carried plasmid YCp2HSE without the *pma1* gene (Δpma1; Table 3), were used as a negative control. In this case, secretory vesicles did not contain ATPase, thus allowing estimation of the level of contamination by plasma membranes carrying the wild-type ATPase synthesized from

Table 2. Cysteine residues in P2 H^+-ATPases of different ascomycetous fungi

Object	Cysteine residue								
	148	221	312	376	409	472	532	569	867
Saccharomyces cerevisiae	+	+	+	+	+	+	+	+	+
Kluyveromyces lactis	+	+	S	+	+	+	+	+	+
Pichia angusta	+	+	A	+	+	+	+	+	+
Pichia stipitis	+	+	+	+	+	+	+	+	+
Vanderwaltozyma polyspora	+	+	G	+	+	+	+	+	+
Candida albicans	+	+	+	+	+	+	+	+	+
Candida glabrata	+	+	+	+	+	+	+	+	+
Zygosaccharomyces rouxii	+	+	+	+	+	+	+	+	+
Schizosaccharomyces pombe	+	+	A	+	+	+	+	+	+
Ashbya gossipii	+	+	+	+	+	+	+	+	+
Debaryomyces hansenii	+	+	+	+	+	+	V	+	+
Neurospora crassa	+	A	S	+	+	+	+	+	+
Yarrowia lipolytica	+	+	T	+	+	+	+	Q	+
Ajellomyces capsulatus	+	A	S	+	+	+	+	S	+
Neosartorya fischeri	+	S	G	+	+	+	+	+	+
Pneumocystis carinii	+	A	A	+	+	+	+	A	+
Aspergillus niger	+	+	S	+	+	+	+	S	+
Aspergillus nidulans	I	+	G	+	A	+	M	+	+
Aspergillus clavatus	I	+	G	+	A	+	M	+	+
Aspergillus fumigatus	+	+	A	+	A	+	M	+	+
Leptosphaeria maculans	+	+	S	+	A	+	M	+	T

Note: Data bank accession numbers for P2-ATPases (from *S. cerevisiae* to *L. maculans*): P05030, P49380, AAD19960, ABN64423, EDO17118, P28877, XM444794, P24545, P09627, AAS554405, CAG84667, P07038, CAG83458, Q07421, EAW21780, AAB06958, CAK43734, AAC27991, EAW09357, AAK94755, CAP70082.

the chromosomal *PMA1* gene. Secretory vesicles were isolated by differential centrifugation and gel filtration [13, 14]. The vesicle fraction was relatively free from plasma membrane contamination (no more than 4%, Δpma1; Table 3) and could readily be used to assay both ATP hydrolysis and proton transport.

Since H^+-ATPase is both a phosphohydrolase and a proton pump, ATP hydrolysis and proton transport were measured in secretory vesicles. It proved possible to monitor the proton-translocating activity because the enzyme active center is exposed at the cytoplasmic surface of secretory vesicles (Fig. 2b), whose membrane has low nonspecific proton permeability. Alanine replacements of any of the seven Cys residues (in positions 148, 221, 312, 376, 532, 569, or 867) did not influence the expression, activity, and kinetics of the enzyme and the coupling of ATP hydrolysis with proton transport. Thus, substitution for these residues did not change the enzyme biogenesis and functioning [13]. However, replacements of Cys221 and Cys532, not affecting the enzyme expression and activity [13], significantly reduced its sensitivity to *N*-ethylmaleimide (NEM) as these residues were the sites of action of this inhibitor [18].

Substitutions of Ala for Cys409 and Cys472 resulted in ATPase activity decrease by 30-40% (Table 3) and K_m increase from 0.7 mM for the wild type enzyme to 1.2 and

1.8 mM for the C409A and C472A mutants, respectively [13], without impairing biogenesis and functioning of the enzyme.

Cys409, Cys472, and Cys376 located in the central catalytic domain were also substituted with Ser; however, only the C409S replacement led to an increase of the mutant enzyme activity compared with the Ala substitution (Table 3). In the case of C472S, the activity was lower and the expression dropped almost twice compared with C472A (Table 3). On substitution of Ser for Cys376, the ATPase lost its activity and only 16% of the newly synthesized enzyme reached secretory vesicles (Table 3). This was in agreement with the data obtained for the Pma1 H^+-ATPase of the *N. crassa* plasma membrane, where replacement of Cys376 with Ser was lethal for the cell [17]. This is apparently due to the fact that Cys376 is followed by Ser377 and Asp378, which is the site of formation of the β-aspartyl phosphate intermediate, and an additional Ser residue makes the enzyme unstable and probably inactive.

Mutants with substitutions for several Cys residues. We replaced simultaneously three to five Cys residues. Substitutions for the NEM-reacting Cys221 and Cys532, along with Cys409, had no effect on the enzyme (C221,409,532A; Table 3). Substitutions for all of the membrane Cys residues, including rather conservative Cys148 and Cys867 and two variable cytosolic Cys221 and Cys569 (C376/409/472/532; Tables 2 and 3), had a minor influence on the enzyme. In the case of another mutant, C376,472,532A containing Cys221, the expression was practically unchanged; however, the activity decreased more than twice (Table 3). The role of residues Cys221, Cys532, and Cys409 will be discussed below.

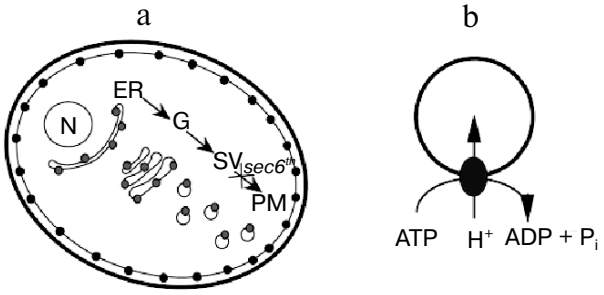

Fig. 2. a) Expression scheme for the Pma1 H^+-ATPase encoded by the plasmid gene *pma1*. The wild type Pma1 H^+-ATPase encoded by the chromosomal gene *PMA1* under the *GAL1* promoter (P_{GAL}-*PMA1*) is synthesized during growth at 23°C on galactose. Secretory vesicles containing the wild type enzyme (black circles) reach plasmalemma and fuse. After shifting of the cells to glucose medium, the synthesis of wild type ATPase is stopped; after elevation of the temperature to 37°C, the enzyme synthesis begins from the plasmid gene *pma1* under the control of heat shock-induced *HSE* promoter (P_{HSE}-*pma1*). Under these conditions, secretory vesicles do not fuse to the plasma membrane. In this case, the vesicles contain ATPase encoded by the plasmid gene *pma1* (gray circles). Abbreviations: N, nucleus; ER, endoplasmic reticulum; G, Golgi apparatus; SV, secretory vesicles; PM, plasma membrane; *sec6*^{ts}, temperature-sensitive mutation *sec*6-4 blocks the fusion of secretory vesicles to the plasma membrane. b) Secretory vesicles house H^+-ATPase transporting protons into the vesicles.

Table 3. Effect of Cys substitutions on the expression and activity of *S. cerevisiae* Pma1 H^+-ATPase (%)

Strain	Expression	ATP hydrolysis	H^+ transport
Wild type	100	100	100
Δpma1	4	3	0
Single substitutions			
C376A	96	82	86
C376S	16	8	0
C409A	81	62	69
C409S	90	84	88
C472A	134	70	71
C472S	79	54	60
Several substitutions			
C221,409,532A	106	98	105
C376,472,532A	91	44	62
C376,409,472,532A	43	9	0
C376, 472,532A/C409S	51	15	17
C376,532A/C409,472S	25	8	0
C221/376/409/472	77	37	–
C376/409/472/532	87	78	81
C221/376/409/472/532	53	57	–

Note: Expression is the amount of Pma1 protein assayed in secretory vesicles. Δpma1, secretory vesicles isolated from the strain carrying plasmid YCp2HSE [13] without the *pma1* gene. Proton transport was expressed as percent of acridine orange fluorescence quenching (% F). Data are the average of 25 experiments for the wild type and of 2-4 for the mutants. 100% corresponded to 4.04 ± 0.26 μmol P_i/min per mg protein (ATP hydrolysis) and 672 ± 47% F/mg protein (H^+ transport).

However, the substitution of Ala for Cys376, Cys409, Cys472, and Cys532 located in the central catalytic domain reduced the mutant expression by half [13], with the activity of ca. 10%. Only when Ser but not Ala was substituted for Cys409 (C376,472,532A/C409S; Table 3), the expression was above 50% and the hydrolytic activity reached 15% allowing the monitoring of proton transport as well. However, when both Cys409 and Cys472 were replaced with Ser, the activity of the mutant enzyme was virtually absent (C376,532A/C409,472S; Table 3). The amount of the mutant Pma1 protein in secretory vesicles decreased fourfold compared with the wild type enzyme (Table 3), and the protein proved to be unstable and easily degradable in spite of the presence of protein inhibitors; low molecular weight proteolysis products were found during electrophoresis (not shown).

Thus, one can conclude that none of the single Cys residues of the Pma1 H^+-ATPase is directly involved in ATP hydrolysis and H^+ transport; however, simultaneous replacement of some of them, especially both Cys409 and Cys472 (Table 3), can significantly impair the enzyme functioning, probably due to the changes in 3D structure and interaction between different domains of the enzyme during the reaction cycle, which involves major conformational changes. Even the replacement of Cys409 only significantly enhanced the enzyme sensitivity to inhibitors: NEM binding to Cys221 and Cys532, and fluorescein-5′-isothiocyanate (FITC) reacting with Lys474 [18].

***One-cysteine* enzymes. Role of Cys409.** Cysteine-less ATPase had no activity and its amount in secretory vesicles was only 20% of the wild type level [13]. However, due to the presence of only one of the nine Cys residues (Cys409), more than half of the mutant C409 enzyme reached vesicles with 24% activity compared with the wild type (Table 4). We have constructed another *one-cysteine* enzyme, C472 (Tables 1 and 4). The presence of this residue in the ATPase molecule was also important for biogenesis and activity of the enzyme, like the presence of Cys409. As seen from Table 4, *one-cysteine* C472 ATPase is characterized by even better expression and higher phosphohydrolytic and proton-translocating activities compared with the C409 enzyme.

Nevertheless, Cys409 appeared to be more important for the normal functioning of the H^+-ATPase than Cys472. It has been shown previously that the replacement of this residue with Ala or Ser changes the sensitivity of the enzyme to inhibitors of different nature: NEM reacting with Cys221 and Cys532, and FITC inhibiting ATPase at Lys474 [18]. The substitution of Ala for Cys409 resulted in a decrease in pK_a of Cys532 (in the mutant C221,409A containing Cys532 but not Cys221) from 9.9 to 8.9 [18]. These data demonstrate that the Ala or Ser substitutions for Cys409 lead to the movement of Cys532 from a hydrophobic (as in the wild type) to a hydrophilic environment. Apparently, the change in pK_a and sensitivity to inhibitors of the enzyme with substituted Cys409 suggests that Cys409 is localized in close proximity to Cys472 and/or Cys532 in the 3D structure [18]. Indeed, Cys409 is surrounded by neutral and polar amino acid residues of sequence 405-MLTACLAAS immediately followed by positively charged motif 414-RKKK. In the enzyme without substitutions, Cys532 is apparently located close to the hydrophobic residues of the LTACLAA sequence; with the Ala/Ser substitution for Cys409, it is shifted to the hydrophilic motif RKKK, which probably accounts for the change in pK_a of Cys532.

Thus, the presence of either Cys409 and/or Cys472 is critical for the biogenesis and functioning of the ATPase. However, both the expression and especially the activity of *one-cysteine* enzymes were low, and it was useful to obtain mutant H^+-ATPases with the minimal cysteine content yet being well expressed and possessing high activity.

***Two-cysteine* enzymes.** Seven different combinations of Ala or Ser substitutions for Cys were tested, with only two Cys residues left in the Pma1 protein (Tables 1 and 4). All of them contained either Cys409 or Cys472 or both. The H^+-ATPase activity of most of them was no higher than half that of the wild type (Table 4). Their expression was close to that of the *one-cysteine* proteins with two exceptions: C312/472 with expression similar to the wild type and C376/472 where expression was lower than that of the *one-cysteine* mutants (Table 4). Only one enzyme containing Cys312 and Cys409 (C312/409) had 55% of the wild type activity; the expression was the same as in the wild type. For two other *two-cysteine* forms (C376/472 and C409/472), the activity was slightly less than half of that of the wild type (Table 4). It is worth mentioning that combination of the critical Cys409 with one of two residues (Cys221 or Cys532) reacting with SH reagents

Table 4. Effect of multiple Cys substitutions on the expression and activity of the *S. cerevisiae* Pma1 H^+-ATPase (%)

Strain	Expression	ATP hydrolysis	H^+ transport
Wild type	100	100	100
Three-cysteine			
C148/409/472	102	68	78
C221/409/532	64	26	41
C312/409/472	117	100	143
C376/409/472	101	79	77
C409/569/867	68	36	37
Two-cysteine			
C148/409	76	33	42
C221/409	65	23	29
C312/409	99	55	58
C409/472	62	49	48
C409/532	67	22	31
C312/472	84	34	–
C376/472	47	47	–
One-cysteine			
C409	55	24	38
C472	77	35	46

Note: Average of 23 experiments for the wild type and of 2-4 experiments for mutants. 100% corresponded to 4.00 ± 0.29 μmol P_i/min per mg protein (ATP hydrolysis) and 777 ± 51% F/mg protein (H^+ transport).

[18] led to a sharp decrease in the ATPase activity, but without substantial effect on its expression and accumulation in secretory vesicles (Table 4). The mutants containing different combinations of replacement of seven out of the nine Cys had no significantly high activity; therefore, we decided to construct *three-cysteine* enzymes (Tables 1 and 4).

***Three-cysteine* enzymes.** Five different mutants carrying Ala substitutions for the six of the nine cysteine residues were constructed (Tables 1 and 4). All of these Pma1 ATPases contained the functionally important Cys409; three of them also had Cys472. The *three-cysteine* C221/409/532 enzyme containing both reactive residues Cys221 and Cys532 had only half of the activity compared with the *two-cysteine* C409/472 (26 and 49% of the wild-type control, respectively; Table 4). By comparison, the enzyme without Cys221 and Cys532 (C221,409,532A) was not different from the wild type (Table 3) but lost sensitivity to NEM [18]. It is worth mentioning that all multiple Cys mutants that contained Cys221 had significantly lower activity compared with those without this residue: e.g. C221/376/409/472 (37%; Table 3) and C376/409/472 (79%; Table 4). The presence of Cys221 also lowered expression except for the C221/409 mutant (Tables 3 and 4). Cys532 did not have such pronounced influence on both expression and activity. Thus, the presence of Cys221 destabilizes the enzyme with multiple substitutions.

The ATPase kinetics of most mutants was insignificantly different compared with the wild type; once again, the enzymes containing either Cys221 or Cys532 showed the most significant difference from the wild type. Nevertheless, *three-cysteine* mutant C221/409/532 had the same affinity to ATP as the wild type control (Table 5). At the same time, the pH optimum was practically the same for the wild type and mutant enzymes: pH 5.7-5.8.

Table 5. Effect of multiple Cys substitutions on kinetics of *S. cerevisiae* Pma1 H^+-ATPase

Strain	K_i, μM	K_m, mM
Wild type	1.8	0.7
Three-cysteine		
C221/409/532	4.1	0.8
C409/569/867	1.3	0.6
Two-cysteine		
C221/409	3.8	1.0
C409/472	2.3	0.7
C409/532	5.4	1.4
One-cysteine		
C409	4.1	0.6

Note: Data are the average of 26 experiments for the wild type and of 2-4 experiments for the mutants.

The expression and activity of another *three-cysteine* enzyme (C409/569/867) were slightly different from those of the *one-cysteine* C409 ATPase (Table 4). Thus, on one hand, these data support the conclusion that at least one of the critical cysteines must be present in the enzyme molecule and, on the other hand, neither cytosolic Cys569 nor membrane Cys867 (in contrast to Cys409) are important for the biogenesis and functioning of Pma1 H^+-ATPase, while the presence of reactive Cys221 has an insignificant effect on biogenesis of the enzyme but significantly reduces its activity. On the contrary, the combinations of critical cysteines with membrane Cys148 and Cys312 and cytosolic Cys376 (in mutants C148/409/472, C312/409/472, and C376/409/472) did not impair ATPase biogenesis, and their phosphohydrolytic and proton-translocating activities were close to those of the wild type. It should be noted that these replacements did not result in a change in coupling between ATP hydrolysis and proton transport compared with substitutions for some charged and polar amino acids, which resulted in overcoupling or uncoupling of H^+-ATPase [19, 20], confirming that Cys residues are not directly involved in proton transport.

Thus, we have obtained and characterized different forms of functionally active ATPase with the minimal cysteine content. Among them, the *three-cysteine* C148/409/472, C312/409/472, and C376/409/472 enzymes are the most promising for further use. The level of their expression and activity is close or slightly different from the wild type. It is important that cysteine residues of these forms are located in different parts of the enzyme: Cys148 in M2, Cys312 in M3, Cys376, Cys409, and Cys472 in the central cytosolic domain. This makes them accessible to some (hydrophobic) and inaccessible to other (hydrophilic) SH reagents. These combinations are plausible for studying the topology and conformational changes during the enzyme reaction cycle. Thereby they can be used both directly and for introduction of new cysteine residues into different enzyme domains in positions of interest for the study of topology and functioning. In particular, *three-cysteine* C376/409/472 ATPase was provided by the author to his colleagues for introducing Cys residues at new positions to study conformational changes during the enzyme functioning; however, the introduced Cys (e.g. Q357C, K372C, A668C, and others) were at the same side of the membrane as the native ones [21]. Therefore, the use of this *three-cysteine* mutant was less effective. The mutants C148/409/472 and C312/409/472 are favorable complements to C376/409/472 as their Cys are located both in membranous and

cytosolic parts of the molecule and they could be used to study function–structure organization of the ATPase without introducing additional Cys residues.

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