Site-directed Mutagenesis of the Yeast PMA1 H\textsuperscript{+}-ATPase

STRUCTURAL AND FUNCTIONAL ROLE OF CYSTEINE RESIDUES

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The yeast plasma-membrane H\textsuperscript{+}-ATPase contains nine cysteines, three in presumed transmembrane segments (Cys-148, Cys-312, and Cys-867) and the rest in hydrophilic regions thought to be exposed at the cytoplasmic surface (Cys-221, Cys-376, Cys-409, Cys-472, Cys-532, and Cys-569). To gather new functional and structural information, we have studied the yeast ATPase by cysteine mutagenesis. It proved possible to replace seven of the nine cysteines by alanine, one at a time, without any significant decrease in ATP hydrolysis or ATP-dependent proton pumping. In the remaining two cases (Cys-409 and Cys-472), there were small but reproducible effects; the results clearly indicated, however, that no single Cys is required for activity and that, if a disulfide bridge is formed in the yeast ATPase, it does not play an obligatory structural or functional role.

Next, multiple mutants were constructed to ask how many Cys residues could be replaced simultaneously while leaving a fully functional enzyme. After substitution of all two “membrane” Cys (Cys-148, Cys-312, and Cys-867) together with two non-conserved Cys located in hydrophilic regions (Cys-221 and Cys-569), there were no significant abnormalities in expression (87%) or activity (89% ATP hydrolysis/93% H\textsuperscript{+} pumping) of the mutant protein. Replacement of two additional cysteines (Cys-376 near the phosphorylation site and Cys-532, in or near the ATP-binding site) caused a drop in expression (to 54%), although the corrected hydrolytic and H\textsuperscript{+} pumping activities were still normal. When Cys-472 was also mutated, the corrected activity fell to 44% hydrolysis/47% pumping; finally, substitution of Cys-409 to give a “cysteine-free” ATPase led to a very poorly expressed and poorly active enzyme. Brief exposure of the “one-cysteine” and “two-cysteine” ATPases to trypsin revealed a normal pattern of degradation, but there was a slight impairment in the ability of vanadate to protect against proteolysis. Thus, although single Cys replacements are tolerated well by the yeast ATPase, multiple replacements are progressively more harmful, suggesting that they cause small but additive perturbations of protein folding.

The P-ATPases are a widespread family of cation transporters, ranging from bacterial K\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Cd\textsuperscript{2+}, and Cu\textsuperscript{2+}-ATPases to the Na\textsuperscript{+}, K\textsuperscript{+}, H\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, and Cu\textsuperscript{2+}-ATPases of mammalian cells (Serrano, 1988; Green, 1992; Fa- gan and Saier, 1994; Bull and Cox, 1994; Soliz et al., 1994). In spite of their physiological diversity, there is clear evidence for a common evolutionary origin. At least 70 P-ATPase genes have been cloned since 1986, and hydroxylation analysis of the corresponding protein sequences has revealed a shared topological plan in which the large catalytic polypeptide is anchored in the membrane by hydrophobic segments at both N- and C-terminal ends (reviewed by Serrano (1988) and Nakamoto et al. (1989)). Within the central hydrophilic portion of the polypeptide are well conserved regions that bind ATP and form the essential \( \beta \)-aspartyl phosphate reaction intermediate. During the past several years, site-directed mutagenesis has made it possible to pinpoint functionally important residues (see, e.g., MacLennan et al. (1992)) and to identify, at least provisionally, the transmembrane segments that form the actual transport pathway (MacLennan et al., 1992; Inesi et al., 1994). In parallel, useful structural information has emerged from cryo-electron microscopy (Green and Stokes, 1992; Toyoshima et al., 1993; Stokes et al., 1994). Much remains to be done, however, before the transport mechanism of the P-ATPases can be completely understood.

With the long range goal of gathering new structural and functional information, we have undertaken to study the yeast plasma-membrane H\textsuperscript{+}-ATPase by cysteine mutagenesis. The yeast ATPase, which is encoded by the PMA1 gene (Ulaszewski et al., 1983; Serrano et al., 1986) and serves as the primary ion pump in the plasma membrane, lends itself well to analysis by both genetic and molecular biological strategies (Cid et al., 1987; McCusker et al., 1987; Nakamoto et al., 1991; Perlin et al., 1992). Furthermore, the yeast enzyme has only nine cysteine residues, unlike its mammalian counterparts which contain 15–33 cysteines (Soliz et al., 1994). Thus, it seemed feasible to start by replacing each of the cysteines in turn with alanine to see which (if any) are required for biogenesis, stability, ATP hydrolysis, and/or ATP-dependent proton pumping. With such information in hand, we could proceed to construct multiple mutants, winding up with a “minimal” Cys ATPase that could be used in a variety of ways: for example, to introduce new Cys residues at structurally or functionally interesting positions where they could be probed by radioactive, fluorescent, or spin-labeled SH reagents. In principle, as shown by work on other membrane proteins, this would pave the way to examine the location, accessibility, and conformational role of individual residues (Falke and Koshland, 1987; Falke et al., 1988; Flitsch and Khorana, 1989; Careaga and Falke, 1992; Pakula and Simon, 1992; Dunten et al., 1993; Frillingos et al., 1994; Lee et al., 1994) and to measure distances between residues in the three-dimensional structure (Wang et al., 1992; Corbalan-Garcia et al., 1993; Baker et al., 1994).

MATERIALS AND METHODS

Preparation of Secretory Vesicles—Strain SY4 of Saccharomyces cerevisiae (MATa; ura3-52; leu2-3, 112; his4-619; sec6-4; GAL; Ura-; Ile-; His-; Leu-; Arg-; Thr-; Trp-)
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pmα1-YFPGal-PMA1 (Nakamoto et al., 1991) was used throughout this work. In SY4, the chromosomal PMA1 gene has been placed under control of the GAL1 promoter (Pgal-PMA1) by the gene disruption strategy of Cid et al. (1987). SY4 also carries a temperature-sensitive mutation in the SEC6 gene, blocking the last step in plasma membrane biogenesis and leading to the accumulation of secretory vesicles (Schein et al., 1989). For secretion assays, the yeast cells were grown to mid-exponential phase (OD600 ~ 0.7-1.0) on supplemented minimal medium containing 2% galactose at 23°C, shifted to medium containing 2% glucose for 3 h to turn off expression of Pgal-PMA1, and then shifted to 37°C for 2 h to turn on expression of P<sub>his</sub>-pmα1.

Secretory vesicles containing newly synthesized ATPase were isolated by differential centrifugation and gel filtration (Nakamoto et al., 1991). To increase the yield of vesicles in the present study, spheroplasts were lysed in 0.4 M sorbitol, 10 mM triethanolamine-acetic acid, pH 7.2, 1 mM EDTA. Protease inhibitors were included in the lysis buffer and in all subsequent steps at the following concentrations: diisopropyl fluorophosphate, 1 mM; chymostatin, 2 μg/ml; and leupeptin, pepstatin, and aprotinin, 1 μg/ml each. After sedimentation at 14,500 x g for 10 min, crude secretory vesicles were resuspended for gel filtration in 0.8 M sorbitol, 10 mM triethanolamine-acetic acid, pH 7.2. Secretory vesicles containing secretory intermediates were collected and centrifuged at 100,000 x g for 45 min, and the vesicles were resuspended at a protein concentration of 0.8-3.0 mg/ml in the same buffer excluding diisopropyl fluorophosphate. The average yield of secretory vesicles was 0.7-0.9 mg of protein per 1000 OD of yeast culture. All preparative procedures were performed at 0-4°C.

Mutagenesis—The Amersham kit for oligonucleotide-directed mutagenesis (Amersham Corp.) was used to introduce mutations into fragments of the PMA1 gene (566 bp, CiaI to BstEII; 615 bp, BstEII to EcoRI; 495 bp, StyI to BamHI; 2149 bp, BamHI to SadI) that had previously been subcloned into a modified version of Bluescript (Stratagene, La Jolla, CA). Each fragment was sequenced to verify the presence of the mutagenesis (and the absence of unwanted base changes, and moved into the full-length PMA1 gene in plasmid pPMa1.2 (Nakamoto et al., 1991). A 3770-bp HindIII to SadI piece containing the entire coding sequence of the gene was then transferred into plasmid YCp2HSE (Nakamoto et al., 1991). The resultant plasmids were transformed into strain SY4 by the method of Itto et al. (1983).

Quantitation of Expressed ATPase—SDS-gel electrophoresis and Western blotting were carried out as described by Nakamoto et al., 1986). The membranes were washed, treated with 1 μCi of [35S]protein A (ICN, Irvine, CA) in 3% bovine serum albumin, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 7.5 mM NaN<sub>3</sub> to block nonspecific antibody-binding sites and then for 6-12 h with 3% bovine serum albumin, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl to block antibody against the four closely related plasma-membrane H<sup>-</sup>-ATPase of Neurospora crassa (Hager et al., 1986). The membranes were washed, treated with 1 μCi of [35S]protein A (ICN, Irvine, CA) in 3% bovine serum albumin, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 7.5 mM NaN<sub>3</sub> for 1-2 h, and assayed for ATPase content in either of two ways: by exposure to Kodak XAR-5 film at −70°C, following by excision of the 100-kDa ATPase band and γ-counting, and/or by means of a PhosphorImager equipped with ImageQuant software version 3.3 (Molecular Dynamics). In either case, the expression of mutant ATPase was calculated relative to a wild-type control run in parallel.

ATPase Activity—ATP hydrolysis was assayed at 30°C in buffer containing 50 mM MES-Tris, pH 7.5, 7.5 mM Na<sub>2</sub>ATP, 5 mM MgCl<sub>2</sub>, 5 mM KCl, and an ATP-regenerating system (5 mM phosphoenolpyruvate and 50 μM pyruvate kinase) in the absence and presence of 100 μM sodium orthovanadate. Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). For determination of pH optimum, the assay medium (above) was preincubated at 30°C, and after preincubation the pH values between 5.0 and 11.0, determined by removing aliquots that were made in the same medium except that Na<sub>2</sub>ATP was replaced with varying amounts of MgATP (1:1); in each case, the true concentration of MgATP was calculated according to Fabiato and Fabiato (1979).

Proton Pumping—H<sup>+</sup> pumping into the secretory vesicles was monitored by fluorescence quenching of the pH-sensitive dye acridine orange, as described previously by Nakamoto et al. (1991) and Rao and Slayman (1993). Assays were carried out at 28°C with continuous stirring on a Hitachi F2000 fluorescence spectrophotometer (excitation, 430 nm; emission, 530 nm) equipped with Intracellular Cation Measurement System software. freshly prepared vesicles (50–100 μg) were suspended in 1.5 ml of 0.6 M sorbitol, 10 mM HEPES-KOH, pH 7.0, 6.0 μM MgCl<sub>2</sub> in the absence or presence of vanadate. After preincubation at 30°C for 3-5 min, trypsin was added to a trypsin:protein ratio of 1.4:1.15 and the incubation continued for 0.5-20 min. Digestion was stopped by the addition of diisopropyl fluorophosphate to a final concentration of 1 mM. To prevent nonspecific proteolysis during electrophoresis, samples were precipitated with trichloroacetic acid (10%), collected by centrifugation, and suspended in SDS-gel loading buffer (Nakamoto et al., 1991).

Protein Assay—Protein concentrations were determined by the method of Lowry et al. (1951) or Bensadoun and Weinstein (1976) using bovine serum albumin as standard.

RESULTS

Replacement of Individual Cys Residues—As illustrated in Fig. 1, the yeast PMA1 ATPase contains nine Cys residues: three in presumed membrane-spanning segments and the remainder in hydrophilic regions that are almost certainly located on the cytoplasmic side of the membrane. Among the nine cysteines, six are conserved among all of the known fungal PMA ATPases: Cys-148 in membrane segment 2; Cys-376, which lies only two residues away from the phosphorylation site (Asp-378); Cys-409; Cys-472, two residues from the site that reacts with fluorescein isothiocyanate (Lys-474; Pardo and Slayman, 1988); Cys-532, close to the conserved "DPPR" motif that is believed to form part of the ATP binding site (Rao et al., 1989; Serrano, 1991); and Cys-867 in membrane segment 10 (Rao and Slayman, 1995).

For reasons outlined in an earlier section, the first step in this project was to replace each of the Cys residues, one at a time, with Ala. In every case, the mutated pmα1 gene was doned downstream of two tandemly arranged heat-shock elements in the expression plasmid YCp2HSE and transformed into yeast strain SY4, in which the resident copy of the PMA1 gene has been placed under control of the GAL1 promoter (Nakamoto et al., 1991). Thus, when the cells were incubated in galactose medium at 23°C, the wild-type ATPase was produced and could support growth; by contrast, when the cells were transferred to glucose medium at 37°C, the mutant ATPase was produced for study. Because strain SY4 carries a temperature-sensitive sec6-4 mutation that blocks the last step in plasma membrane biogenesis (Scheinman and Novick, 1982), the shift to 37°C also led to the accumulation of secretory vesicles containing the newly synthesized mutant ATPase. The secretory vesicles were isolated by differential centrifugation followed by size-fractionation on a Sephacryl column (Walworth and Novick, 1987). They were relatively free of contamination by plasma membrane fragments containing pre-existing wild-type ATPase (Nakamoto et al., 1991; see also Table I), and could readily be assayed for ATP hydrolysis and ATP-dependent H<sup>+</sup> pumping (the latter, by acridine orange fluorescence quenching; Nakamoto et al., 1991).

Results for the nine Cys→Ala mutants are summarized in Table I. In every case, the measured expression level was 80% or greater of the wild-type control, indicating that all nine mutant ATPases were synthesized properly, moved from the endoplasmic reticulum to the secretory vesicles, and remained stable over the time course of the experiment. Similarly, all of the mutant proteins had relatively high ATPase activities and ATP-dependent H<sup>+</sup> pumping rates. After correction for the

1 The abbreviations used are: bp, base pair(s); MES, 2-(N-morpholino)ethanesulfonic acid.
expression level, values ranged from 55% hydrolysis/53% pumping in the C472A mutant and 74% hydrolysis/81% pumping in the C409A mutant to 113% hydrolysis/119% pumping in C312A. No significant abnormalities were detected in the kinetic parameters of ATP hydrolysis, including the $K_m$ for ATP (0.6–1.8 mM), $K_i$ for orthovanadate (1.1–3.1 mM), and pH optimum (5.6–5.9). Thus, it appears that none of the nine Cys residues plays an essential role in the biogenesis or functioning of the yeast PMA1 H$^+$-ATPase.

Because Ser is sometimes better than Ala at substituting for Cys, especially in relatively hydrophilic regions of proteins, Cys $\rightarrow$ Ser replacements were also constructed for Cys-409 and Cys-472 (Table I). Indeed, the C409S ATPase had marginally higher hydrolytic and pumping activities (94%/99% after correction for expression levels, compared with 74%/81% for the C409A ATPase). The same was true for the C472S enzyme (74%/78% compared with 55%/53% for the C472A enzyme), although in the latter case the Ser replacement gave a noticeably lower expression level (75%) than the Ala replacement (134%). In any event, the Ser substitutions provided an added option for the design and characterization of multiple mutants (see below).

**Replacement of Multiple Cys Residues**—The next step was to ask how many Cys residues could be replaced simultaneously without any major effect on expression, ATP hydrolysis, or proton pumping. For this purpose, multiple mutants were constructed in several stages either by site-directed mutagenesis or by combining fragments carrying single or multiple cysteine substitutions (Table II). In strain C148A/C221A/C312A, for example, two Cys residues in membrane segments 2 and 3 and a third Cys in the small hydrophilic loop between segments 2 and 3 were replaced with alanine. The resulting ATPase was expressed at a relatively high level in secretory vesicles (77%), hydrolyzed ATP at 103% of the wild-type rate, and carried out ATP-dependent proton pumping at 75% of the wild-type rate; it had no significant abnormalities in $K_m$ for MgATP, $K_i$ for vanadate, or pH optimum (Table III). Strain C148A/C221A/C312A/C569A/C867A contained two additional replacements,
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For details, see Table I and “Materials and Methods.” This series of experiments included 11 preparations of wild-type with an average ATPase activity of 4.51 ± 0.30 μmol P_i/min · mg and an average quenching of 676 ± 80% Q/mg. Mutant values are the averages of at least two determinations, each corrected relative to a parallel wild-type control.

Table II

| Mutant                              | Replacement                | Uncorrected | Corrected | K_m | K_i | pH optimum | H- transport |
|-------------------------------------|----------------------------|-------------|-----------|-----|-----|------------|-------------|
| Wild type                           |                            | 100%        |           | 4.51 |     | 0.7       | 1.8         | 5.7         | 676         |
| C148A/C221A/C312A                   | A                          | 77%         | 103%      | 3.86 | 0.7 | 1.3       | 1.1         | 5.7         | 439         |
| C148A,221,312,569,676A              | A                          | 87%         | 89%       | 3.85 | 0.9 | 0.9       | 0.9         | 5.7         | 745         |
| C409A/C472A                        | A                          | 93%         | 44%       | 2.48 | 0.7 | ND*       | 5.6         |             | 301         |
| C409A/C472A                        | A                          | 48%         | 50%       | 1.21 | 0.6 | 3.8       | 5.8         |             | 148         |
| C376A/C409A/C472A/C532A            | A                          | 43%         | 21%       | 0.40 | ND  | ND        | ND          |             | 207         |
| C376A/C407A/C472A/C532A/C409S      | A                          | 51%         | 30%       | 0.58 | ND  | ND        | ND          |             | 207         |
| C376A/C532A/C409S/C472S            | A                          | 25%         | 23%       | 0.29 | ND  | ND        | ND          |             | 207         |
| Two-cysteine                       | A                          | 54%         | 103%      | 2.09 | 0.8 | 2.3       | 5.8         |             | 439         |
| One-cysteine                       | A                          | 55%         | 44%       | 0.85 | 0.6 | 4.1       | 5.7         |             | 259         |
| Cysteine-free                      | A                          | 20%         | 33%       | 0.30 | ND  | ND        | ND          |             | 207         |

a ND, not determined.

Effect of multiple cysteine replacements on PMA1 ATPase

To pave the way for future studies, it seemed worthwhile to characterize the two-cysteine and one-cysteine ATPases in greater detail. As illustrated in Fig. 2, both enzymes displayed K_m values for MgATP (0.8 and 0.6 μM) very close to the wild-type value, suggesting a relatively normal ability to bind MgATP and proceed through the early portion of the catalytic cycle. Likewise, the two-cysteine mutant had a virtually unchanged K_i for vanadate (2.3 μM compared with 1.8 μM in the wild type), while in the one-cysteine mutant, the K_i rose slightly to 4.1 μM; the latter change may reflect a minor impairment of the vanadate-binding site or alternatively a slight shift in favor of the vanadate-insensitive E2 conformation. Both mutants pumped protons as well as one would predict based on their rates of ATP hydrolysis (corrected values of 97% versus 103% in the two-cysteine enzyme; 47% versus 44% in the one-cysteine enzyme; see also Fig. 3), ruling out any significant change in energy coupling ability.

Limited Trypsinolysis—To examine the conformational state of the mutant ATPases directly, the wild-type, two-cysteine, and one-cysteine enzymes were subjected to limited trypsinolysis. In the experiment of Fig. 4, secretory vesicles were incubated at a trypsin:protein ratio of 1:15 for 0–20 min. At each one at position 569 (a non-conserved Cys toward the end of the central hydrophilic loop) and the other at position 867 (in membrane segment 10). Once again, expression (87%), ATP hydrolysis (89%), and ATP-dependent proton pumping (93%) were virtually unchanged, as were the measured kinetic parameters.

A parallel set of constructs explored the consequences of combining mutations within the central hydrophilic region. The double mutant C409A/C472A was expressed normally (93%) but had reduced rates of ATP hydrolysis and ATP-dependent proton pumping (44% and 46%, respectively), some-what below the values seen with the corresponding single mutants (C409A, 74% and 81%; C472A, 55% and 53%; Table I). Serine replacements at the same positions (in C409S/C472S) were less successful, giving corrected hydrolytic and pumping rates of 50% and 58% but lowering expression to 48% (Table III). When mutations were added at two neighboring sites to give constructs with four alanines (C376A/C409A/C472A/C532A), three alanines and one serine (C376A/C472A/C532A/C409S/C472S), expression occurred at 43%, 51%, and 25% of the wild-type level, respectively. Even after correction, hydrolytic rates were quite low (21%, 30%, and 32%); ATP-dependent proton pumping was detectable only in the middle case (45%; Table III).

The last step was to combine mutations throughout the ATPase. In a "two-cysteine" strain containing only Cys-409 and Cys-472, expression was acceptable (54%) and the corrected rates of ATP hydrolysis and ATP-dependent proton pumping were essentially normal (103% and 97%). When Cys-472 was replaced with Ala, leaving only Cys-409 ("one-cysteine" strain), expression remained at 55% but hydrolysis and pumping dropped to 44% and 47%. And finally, when Cys-409 was replaced with Ser to give a "cysteine-free" enzyme, expression fell to 20%. ATP was now hydrolyzed at a barely measurable rate (6% before correction; 33% after correction), and no ATP-dependent proton pumping was seen. Taken together, the results point to additive effects of multiple cysteine substitutions, even though the replacement of any single Cys can be tolerated quite well. Indeed, it appears impossible to achieve a properly functioning H^+-ATPase that is entirely cysteine-free, even though such an enzyme is capable of splitting ATP at a very slow rate. By contrast, mutant enzymes with either two cysteines (Cys-409 and Cys-472) or one cysteine (Cys-409) are active enough to serve as a useful starting point for further work.

Table III

| Mutant | Expression | ATPase | K_m | K_i | pH optimum | H^+ transport |
|--------|------------|--------|-----|-----|------------|---------------|
| Wild type | 100%      | 4.51 | 0.7 | 1.8 | 5.7        | 676           |
| C148A/C221A/C312A | 77%       | 3.86 | 1.3 | 1.1 | 5.7        | 439           |
| C148A,221,312,569,676A | 87%       | 3.85 | 0.9 | 0.9 | 5.7        | 745           |
| C409A/C472A | 93%       | 2.48 | 0.7 | ND* | 5.6        | 301           |
| C409A/C472A | 48%       | 1.21 | 0.6 | 3.8 | 5.8        | 148           |
| C376A/C409A/C472A/C532A | 43%       | 0.40 | 0.3 | ND  | ND        | 207           |
| C376A/C407A/C472A/C532A/C409S/C472S | 51%       | 0.58 | 0.7 | ND  | ND        | 207           |
| C376A/C532A/C409S/C472S | 25%       | 0.29 | 0.6 | 4.1 | 5.7        | 259           |
| Two-cysteine | 54%       | 2.09 | 0.8 | 2.3 | 5.8        | 439           |
| One-cysteine | 55%       | 0.85 | 0.6 | 3.8 | 5.7        | 259           |
| Cysteine-free | 20%       | 0.30 | ND  | ND  | ND        | 207           |

a ND, not determined.
time point, the reaction was stopped by the addition of diisopropyl fluorophosphate (1 mM), and the products of digestion were separated by SDS-gel electrophoresis and blotted with anti-ATPase antibody. Under these conditions, the 100-kDa wild-type protein was cleaved rapidly (within 0.5 min; data not shown) to yield a 97-kDa form, followed by the slower appearance of 62- and 48-kDa fragments. The same pattern was seen for the two-cysteine and one-cysteine ATPases, except that the smaller fragments appeared to be marginally less stable (Fig. 4).

Further information came from digesting with trypsin in the presence of vanadate, which has previously been shown to protect the yeast PMA1 ATPase against proteolytic degradation (Perlin and Brown, 1987). With both the wild-type and mutant enzymes, as little as 1 μM vanadate was sufficient to give visible protection of the initial 97-kDa cleavage product (Fig. 5). However, quantitative scanning of the immunoblot revealed that the extent of protection was noticeably lower in the mutants (~30–40%) than in the wild type (~70%). Thus, the elimination of seven cysteines or eight cysteines led to minor changes in conformation, making the ATPase more sensitive (or more accessible) to the action of proteolytic enzymes.

**DISCUSSION**

As shown in this study, it is possible to substitute each of the nine cysteines of the yeast PMA1 ATPase singly without a major effect on expression, ATP hydrolysis, or ATP-dependent proton pumping; we therefore conclude that no individual Cys residue is essential for the biogenesis or functioning of the enzyme. This is not a surprising result in the case of the three non-conserved cysteines (Cys-221, Cys-312, and Cys-569). The remaining six cysteines are found in all known fungal H^+\text{-ATPases}, however, and at least in principle might have been predicted to play an important functional role.

Indeed, working with the closely related *Neurospora* plasma-membrane H^+\text{-ATPase}, Rao and Scarborough (1990) have put forward biochemical evidence that two membrane-embedded cysteines may be linked by a disulfide bridge. Disulfides have also been reported in the sarcoplasmic reticulum Ca2^+\text{-ATPase} (Thorley and Green, 1977) and the gastric H^+,K^+\text{-ATPase} (Sachs et al., 1992), and the latter enzyme is inhibited by formation of a disulfide bridge with the anti-ulcer drug omeprazole (Prinz et al., 1992). Several functions can be imagined for intramolecular disulfide bridges: for example, to help in the assembly of the nascent ATPase polypeptide or to stabilize the mature molecule (Rao and Scarborough, 1990). Alternatively, disulfide bridge formation might mediate reversible activation/deactivation, as has been described for the vacuolar H^+\text{-ATPase} from clathrin-coated vesicles (Feng and Forgac, 1994).

In the case of the yeast PMA1 ATPase, however, molecular biological evidence now argues quite convincingly against the presence of an essential disulfide bridge. With respect to membrane-embedded cysteines, Harris *et al.* (1991) reported that...
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Cys-148 can be replaced by Ser with little or no effect on ATPase activity, and the present study has shown that substitution of Cys-148, Cys-312, or Cys-867 singly, or even substitution of all three simultaneously (in strain C148A/C221A/C312A/C569A/C676A; Tables II and III), produces no significant change in biogenesis or activity. Thus, if a disulfide bridge is formed in the membrane portion of the yeast ATPase, it cannot play an obligatory structural or functional role, at least under the conditions of these experiments. The situation with respect to the six cytoplasmically located cysteines is a little more complicated, since there were significant effects of multiple Cys replacements in this region. Once again, however, no single Cys substitution caused a major impairment of ATPase biogenesis or function, arguing that no disulfide bond can be absolutely essential. Consistent with this idea, ATPase activity in secretory vesicles is not inhibited by the presence of 10 mM dithiothreitol or 28 mM 2-mercaptoethanol in the phosphorylation domain, while the other two (Cys-472 and Cys-478) are little more complicated, since there were significant changes in biogenesis or activity. Thus, if a disulfide bond can be absolutely essential. Consistent with this idea, ATPase activity in secretory vesicles is not inhibited by the presence of 10 mM dithiothreitol or 28 mM 2-mercaptoethanol (Georgoussi and Sotiroudis, 1985; Mutoh et al., 1990).

Effect of vanadate on trypsinolysis of wild-type and mutant ATPases. Secretory vesicles (10 μM of protein) containing wild-type (lanes 1–6), two-cysteine (lanes 7–12), or one-cysteine (lanes 13–18) ATPase were incubated for 7.5 min at a trypsin:protein ratio of 1:15 in the presence of 0–100 μM vanadate. For experimental details, see “Materials and Methods.”

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REFERENCES

Baker, J. K., East, J. M., and Lee, A. G. (1994) Biochim. Biophys. Acta 1219, 53–60.
Bensadoun, A., and Weinstein, A. (1991) J. Biol. Chem. 266, 241–250.
Bull, P. C., and Cox, D. W. (1994) Trends Genet. 10, 246–252.
Careaga, C. L., and Falke, J. J. (1992) Biochim. Biophys. Acta 1125, 209–219.
Cid, A., Perona, R., and Serrano, R. (1987) Curr. Genet. 12, 105–110.
Corbalan-Garcia, S., Teruel, J., and Gomez-Fernandez, J. C. (1993) Eur. J. Biochem. 217, 737–744.
Daiho, T., and Kanazawa, T. (1994) J. Biol. Chem. 269, 11060–11064.
Dibner, R. L., Sahin-Toth, M., and Kaback, H. R. (1993) Biochemistry 32, 12644–12650.
Fabio, A., and Fabio, F. (1979) J. Physiol. 275, 463–505.
Fagan, M. J., and Sailer, M. H. (1994) J. Biol. Chem. 269, 57–99.
Falke, J. J., and Koshland, D. E., Jr. (1987) Science 237, 1596–1600.
Falke, J. J., Dengburg, A. F., Sternberg, D. A., Zalkin, N., Milligan, D. L., and Koshland, D. E., Jr. (1988) J. Biol. Chem. 263, 4950–4958.
Feng, Y., and Foragc, M. (1994) Biochem. Biophys. Res. Commun. 202, 133–137.
Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
Flitsch, S. L., and Khorana, H. G. (1989) Anal. Biochem. 183, 265–275.
MacLennan, D. H., Clarke, D. M., Lou, T. W., and Skjerjanec, I. S. (1992) Acta Physiol. Scand. 146, (Suppl. 607), 141–150.
Makamoto, R., R., and Slayman, C. W. (1991) J. Biol. Chem. 266, 7940–7949.
Nakamoto, R. K., R., and Slayman, C. W. (1991) J. Biol. Chem. 266, 7940–7949.
Pakula, A. A., and Simon, M. I. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4128–4132.
Parde, J. P., and Slayman, C. W. (1988) J. Biol. Chem. 263, 18664–18668.
Perlin, D. S., and Brown, C. L. (1987) J. Biol. Chem. 262, 6788–6794.
Perlin, D. S., Harris, S. L., Monk, B. C., Sato-Young, D., Na, S., Anand, S., and Kaback, H. R. (1992) Acta Physiol. Scand. 146, (Suppl. 607), 183–192.
Petri, V., Smirnova, M. V., and Okorokov, L. A. (1993) J. Biol. Chem. 268, 2463–2465.
Prinzip, C., Kajimura, M., Scott, D., Helander, H., Shin, J., Besancon, M., Bamberg, K., Herbry, S., and Sachs, G. (1992) J. Biophys. 87, 259–266.
Rao, S. U., and Scarbrough, G. A. (1990) J. Biol. Chem. 265, 7227–7235.
Rao, S., and Slayman, C. W. (1993) J. Biol. Chem. 268, 6708–6713.
Rao, R., and Slayman, C. W. (1993) J. Biol. Chem. 268, 6708–6713.
Sachs, G., Besancon, M., Shin, J. M., Mercier, F., Munson, K., and Hersey, S. (1992) J. Bionerg. Biomembr. 24, 301–308.
Scheele, R., and Novik, R. (1990) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. R., Pringle, J. R., and Jones, E. W., eds) pp. 523–585, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28.
Serrano, R. (1991) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Genomes Dynamics, Protein Synthesis, and Energistics (Broach, J. R., Pringle, J. R., and Jones, E. W., eds) pp. 523–585, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Serrano, R., and Portillo, F. (1990) Biochim. Biophys. Acta 1018, 195–199.
Shewan, J., Kielland-Brandt, M., and Fink, G. R. (1986) Nature 319, 689–693.
Soliz, M. G., Odermatt, A., and Krapf, R. (1994) FEBS Lett. 346, 44–47.
Stokes, D. L., Taylor, W. R., and Green, N. M. (1994) FEBS Lett. 346, 32–38.
Thuery-Lawson, D. A., and Green, N. M. (1997) Biochem. J. 327, 739–748.
Wilding, C., Cassab, H., and Stokes, D. L. (1993) Nature 362, 467–471.
Ulaszewski, S., Grenson, M., and Goffaux, A. (1983) Eur. J. Biochem. 130, 235–239.
Walworth, N. C., and Novick, P. J. (1987) J. Cell. Biol. 105, 163–174.
Wang, Z., Gergely, J., and Tao, T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11811–11817.

2 V. V. Petrov, unpublished experiments.