Mutations in the CYS4 Gene Provide Evidence for Regulation of the Yeast Vacuolar H^+-ATPase by Oxidation and Reduction in Vivo*

Yemisi E. Oluwatosin and Patricia M. Kane‡

From the Department of Biochemistry and Molecular Biology, State University of New York Health Science Center at Syracuse, Syracuse, New York 13210

The vma41-1 mutant was identified in a genetic screen designed to identify novel genes required for vacuolar H^+-ATPase activity in Saccharomyces cerevisiae. The VMA41 gene was cloned and shown to be allelic to the CYS4 gene. The CYS4 gene encodes the first enzyme in cysteine biosynthesis, and in addition to cysteine auxotrophy, cys4 mutants have much lower levels of intracellular glutathione than wild-type cells. cys4 mutants display the pH-dependent growth phenotypes characteristic of vma mutants and are unable to accumulate quinacrine in the vacuole, indicating loss of vacuolar acidification in vivo. The vacuolar proton-translocating ATPases (V-ATPase) is synthesized at normal levels and assembled at the vacuolar membrane in cys4 mutants, but its specific activity is reduced (47% of wild type) and the activity is unstable. Addition of reduced glutathione to the growth medium complements the pH-dependent growth phenotype, partially restores vacuolar acidification, and restores wild type levels of ATPase activity. The CTS4 gene was deleted in a strain in which the catalytic site cysteine residue implicated in oxidative inhibition of the yeast V-ATPase has been mutated (Liu, Q., Leng, X.-H., Newman, P., Vasilyeva, E., Kane, P. M., and Forgac, M. (1997) J. Biol. Chem. 272, 11750–11756). This catalytic site point mutation suppresses the effects of the cys4 mutation. The data indicate that the acidification defect of cys4 mutants arises from inactivation of the vacuolar ATPase in the less reducing cytosol resulting from loss of Cys4p activity and provide the first evidence for the modulation of V-ATPase activity by the redox state of the environment in vivo.

Many organelles of the eukaryotic cell network, including the vacuoles/lysosomes, Golgi apparatus, endosomes, clathrin-coated vesicles, synaptic membrane vesicles, chromaffin granules, and other secretory vesicles, are acidified by a single class of proton pumps, the vacuolar proton-translocating ATPases (V-ATPases). V-ATPases are multi-subunit complexes with an overall structure and subunit composition very similar to the F_0F_1-ATPases of bacteria, chloroplasts, and the inner mitochondrial membrane. The V_1 sector of the V-ATPase, which contains the ATP-binding sites, is a cytoplasmically-oriented complex of peripheral subunits, while the V_0 sector consists of integral membrane subunits and contains the proton pore. The electrochemical gradient generated by the V-ATPases is crucial for processes such as protein sorting, zymogen activation, receptor-mediated endocytosis, and the transport of ions, amino acids, and other metabolites.

V-ATPases are highly conserved between fungi, plants, and animals. Thirteen different polypeptides, ranging in molecular mass from 10 to 100 kDa have been identified as subunits of the V-ATPase of the yeast Saccharomyces cerevisiae. The genes encoding all of these subunits have been cloned (Ref. 6 and references therein, see also, Refs. 7 and 8). The products of four other genes (VMA12, VMA21–23) are also required for assembly of the yeast V-ATPase even though they are not part of the final complex (9–11).

V-ATPases are present in several distinct locations within a single cell. Very little is known about how enzyme activity is regulated in vivo to maintain different organelles within a single cell at different specific pH values or to adjust organelle acidification in response to changing extracellular conditions. Several mechanisms have been proposed for the regulation of vacuolar acidification by V-ATPases (see Ref. 12 for a recent review). Reversible dissociation of the peripheral V_1 and integral V_0 domains in response to changes in growth conditions (13, 14) have indicated that disassembly and reassembly may be a means of regulating V-ATPase activity in vivo. Regulation of acid secretion by changes in the density of V-ATPase in the apical membrane has been demonstrated in intercalated cells in the kidney (15). Reversible disulfide bond interchange (16–18), changes in the degree of coupling between ATP hydrolysis and proton pumping (19, 20), and changes in membrane potential (21, 22) have also been suggested as possible means of regulating V-ATPase activity.

Biochemical studies on the enzyme isolated from bovine clathrin-coated vesicles have indicated that reversible sulfhydryl-disulfide bond interconversion within the catalytic subunit may play a role in controlling V-ATPase activity in vivo (16–18). Specifically, these studies show that disulfide bond formation between conserved cysteine residues near the nucleotide-binding site of the catalytic subunit results in inactivation of the V-ATPase and that this inactivation can be reversed by a disulfide interchange within the catalytic subunit. Furthermore, Deschida and Bowman (23) showed that reducing agents have a stabilizing effect on the V-ATPase from Neuro-

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† American Heart Association Established Investigator. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, SUNY Health Science Center at Syracuse, 750 E. Adams St., Syracuse, NY 13210. Tel.: 315-464-8742; Fax: 315-464-8750; E-mail: kanepm@vaex.cs.hescyr.edu.

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; V_1, peripheral sector of the yeast vacuolar H^+-ATPase; V_0, integral membrane sector of the yeast vacuolar H^+-ATPase; SD-ura (or SD-leu, SD-met), supplemented minimal medium containing 2% dextrose lacking uracil (or leucine or methionine); GSH, reduced glutathione; GSSG, oxidized glutathione; MES, 2-(N-morpholino)ethanesulfonic acid; NEM, N-ethylmaleimide; DTT, dithiothreitol; kb, kilobase(s); MOPS, 4-morpholinopropanesulfonic acid.

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spora crassa and oxidizing agents are potent inhibitors of the V-ATPase in vitro. These results suggest that the redox state of the immediate environment may be an important regulator of vacuolar ATPase activity, but this has not been demonstrated in vivo.

In a genetic screen designed to identify novel genes affecting V-ATPase activity, we isolated a mutation in the CYS4 gene. We report here that the product of the CYS4 gene is required for the in vivo activity, but not the biosynthesis and assembly of the yeast vacuolar H⁺-ATPase, when cells are grown in rich medium. Mutations in CYS4 lead to a decrease in the cellular concentration of reduced glutathione as a result of impaired cysteine biosynthesis. Our results provide the first in vivo evidence in support of previous results (17, 23) which suggest that the V-ATPase may be inactivated in a less reducing environment and that this inactivation involves a highly conserved catalytic site cysteine.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were purchased from New England Biolabs and Boehringer Mannheim. Taq DNA polymerase was purchased from Boehringer Mannheim. Zymolyase 100T was purchased from Watanabe Biochemical Products. 1-kb DNA ladder and precast protein molecular mass standards were obtained from Life Technologies, Inc. Synthetic oligonucleotide primers for polymerase chain reaction and sequencing were obtained from Genosys. Zwittergent 3–14 (ZW3–14) was obtained from Calbiochem. All other reagents were purchased from Sigma.

Methods—Removal of vacuolar membrane vesicles were performed as described by Sambrook (29). Indirect immunofluorescence microscopy and preparation of antibodies were performed as described (3). Spores in which a vacuolar phenotype was observed were selected by their cysteine auxotrophy. Yeast genomic DNA was isolated from chromosomal DNA using synthetic oligonucleotides 5'-GGATAGATCCTACCCCTGCA-3' and 5'-GATACCATGTTGAGCTAC-3'. Isolation of yeast genomic DNA for polymerase chain reaction analysis was carried out as described by Nasmyth and Reed (32) except that DNA was treated with RNase A for 25 min at 37 °C and 5 min at 65 °C before the final precipitation.

Tetrad Analysis—Haploid yeast strain YOY14-4Ba carrying the CYS4 gene on a URA3-containing plasmid (pYO38) was mated with haploid strain SF838-5Aa cy4Δ LEU2. Diploids were selected on supplemented minimal medium lacking both uracil and leucine and named YOY12/pYO38. YOY12/pYO38 cells growing in YEPD, pH 5.0, plates were patched on sporulation medium and incubated at 30 °C for 5–6 days. Tetrad were dissected on YEPD, pH 5.0, plates and incubated at 30 °C for 48 h to allow the spores to germinate.

Construction of the YOY13-2Ca Strain—The CYS4 locus was disrupted in a vma1Δ::URA3 strain (SF838-5Aa vma1–51) as described above. The resulting vma1Δ cy4Δ strain was crossed to yeast strain PNY1 (33) to obtain a heterozygous diploid yeast strain (YOY13) with only one functional copy of the CYS4 gene and no wild-type allele of the VMA1 gene (the only functional copy is the C261V mutant allele, designated here as vma1–51, integrated at the URA3 locus). YOY13 was sporulated and tetrads were dissected on YEPD, pH 5.0, plates. cy4Δ spores, identified by their cysteine auxotrophy, were selected. Whole cell lysates were prepared from selected (cy4Δ) spores and analyzed by Western blotting using monoclonal antibody 7D5 directed against the 69-kDa VMA1 gene product. This antibody is able to detect the product of the vma1–51 mutant allele of VMA1 (33). Spores in which the vma1–51 mutation had co-segregated with the cy4Δ mutation were selected. One such spore, YOY13-2Ca, was used in the experiments described here.

Quinacrine Vital Staining—Vaccumulation of quinacrine was assessed as described by Roberts et al. (34). Once stained, cells were visualized within 10 min using a Zeiss Axioskop Routine immunofluorescence microscope. Cells were viewed under Nomarski optics to observe normal cell morphology and under a fluorescein isothiocyanate filter with a 10× objective to observe vacuolar staining.

Purification of the Yeast V-ATPase—Solubilization of vesicles and purification of the vacuolar H⁺-ATPase were performed basically as described (3, 51) with the following modifications. 0.5–1 mg of solubilized vesicles were layered on a 12 ml of 20–50% (w/v) glycerol gradient and centrifuged at 200,000 × g for 8 h in a Beckman Ti-75 rotor. Sixteen 700-μl fractions were collected and analyzed for ATPase activity to identify fractions containing peak ATPase activity. Fractions were diluted 1:1 with water and protein precipitated by addition of an equal volume of 20% trichloroacetic acid. Precipitated proteins were solubilized in 50 μl of cracking buffer (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 8 μl urea, 5% SDS, 5% β-mercaptoethanol), separated on a 10% SDS-polyacrylamide gel, and detected by silver staining or Western blotting. To determine the specific activity of the purified V-ATPase protein was precipitated as described above and resuspended in cracking buffer lacking β-mercaptoethanol. Protein concentration was then determined using Bio-Rad DC Protein Assay kits.

Western Blotting—Whole cell lysates and solubilized vacuolar membrane vesicles were prepared, and SDS-polyacrylamide gel electrophoresis was performed as described previously (35). Immunoblots were probed with monoclonal antibodies 10D7, 7D5, 13D11, and 7A2, and probed with monoclonal antibodies 10D7, 7D5, 13D11, and 7A2, and 27-kDa subunits, respectively, of the yeast V-ATPase (35, 36). Bound antibodies were detected using alkaline phosphatase-conjugated secondary antibodies.

DNA Sequencing—Plasmid DNA for sequencing was purified using the QIAprep-spin Plasmid Kit from QIAGEN. Sequencing was done by the dyeoxy chain termination method using Sequenase® sequencing kit with Sequenase version 2.0 (U. S. Biochemical Corp.) and 32P-dATP.

Other Methods—Indirect immunofluorescence microscopy and preparation of vacuolar membrane vesicles were performed as described by

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**Table I**

| Name      | Genotype          | Ref. |
|-----------|-------------------|------|
| SF838-5Aa | MATα ura3-52 leu2-3,112 his4-419 ade6 | 57   |
| YOY14-4Ba | MATα ura3-52 leu2-3,112 his4-419 ade6pp4-3 vma1-1 | 48   |
| SF838-5Aa cy4Δ | MATα ura3-52 leu2-3,112 his4-419 ade6 cy4Δ::LEU2 | 48   |
| NO11-2    | MATα ura3-52 leu2-3,112 his4-419 ade6 vma1::LEU2 URA3::VMA1 | 32   |
| PNY1      | MATα ura3-52 leu2-3,112 his4-419 ade6 vma1::LEU2 URA3::vma1–51 | This study |
| YOY13-2Ca | MATα ura3-52 leu2-3,112 his4-419 ade6 vma1::LEU2 URA3::vma1–51 cy4Δ::LEU2 | This study |
| YOY12     | YOY14-4Ba X SF838-5Acy4Δ | This study |

* Footnote 2.
Regulation of V-ATPase by Oxidation and Reduction

Identification of the VMA41 Gene as CYS4—The MEY14 strain, carrying the vma41-1 mutation was obtained in a genetic screen designed to identify genes required for vacuolar membrane ATPase activity in yeast. The screen was based on the set of growth phenotypes characteristic of mutants with loss of vacuolar membrane V-ATPase activity (vma mutants). The Vma<sup>−</sup> growth phenotypes include inability to grow in medium buffered to pH 7 or above, medium containing 100 mM CaCl<sub>2</sub> or medium containing a non-fermentable carbon source. After backcrossing to the original MEY14 strain to remove background mutations, one Vma<sup>−</sup> spore, YOY14-4Ba, was selected for further analysis. In addition to Vma<sup>−</sup> phenotypes, both the MEY14 mutant and the YOY14-4Ba strain are unable to grow on minimal medium, suggesting the cosegregation of the Vma<sup>−</sup> phenotype and an undetermined nutritional auxotrophy. The VMA41 gene was cloned by complementation of the pH-dependent growth phenotype of the YOY14-4Ba strain. The YOY14-4Ba mutant strain was transformed with a yeast DNA library on a single copy (CEN) plasmid (YCP50). Transformants were selected on SD-ura, pH 7.5, medium and 15 independent Ura<sup>−</sup> Vma<sup>+</sup> transformants were obtained. Plasmids were recovered from transformants and re-checked for complementation. All 15 plasmids were able to restore growth at pH 7.5 to the YOY14-4Ba strain. Restriction endonuclease analyses revealed that all 15 contain the same 11-kb yeast DNA insert (Fig. 1). Various subclones of plasmid pMEY14-1 were generated in the yeast shuttle vector pRS316. Analyses of these subclones indicated that a 2-kb XbaI-SphI fragment is sufficient for complementation (Fig. 1). A 350-base pair region internal to this fragment was sequenced and used to search for homology to any sequences in the GenEMBL data base. This analysis revealed that the sequenced region (indicated by the arrows in Fig. 1) lies within the reported nucleotide sequence of the yeast NH55 gene for β-thionase, also known as the STR4 or CYS4 gene for cystathionine-β-synthase (40, 41). For clarity, the CYS4 nomenclature is used throughout this report. The CYS4 gene product is essential for cysteine metabolism; as a result, cys4 mutants exhibit a cysteine-dependent growth phenotype (40). A LEU2-disrupted copy (plasmid pYO50) of the CYS4 open reading frame was constructed as shown in Fig. 1. Haploid yeast strain SP38-5As was transformed with Apal/SalI-digested pYO50 and stable Leu<sup>+</sup> transformants selected. DNA was extracted from three independent transformants and the parental wild type strain and analyzed by polymerase chain reaction to confirm the disruption of the CYS4 locus in the transformants. The results (not shown) indicate that the 968-base pair fragment, expected from the wild type CYS4 locus, was replaced by a larger, 2.3-kb fragment in the transformants, indicating that the CYS4 gene has been disrupted in these cells. The growth phenotypes of cys4Δ cells are indistinguishable from those of the original YOY14-4Ba mutant. In addition to the characteristic Vma<sup>−</sup> growth phenotypes, cys4Δ cells are unable to grow on minimal medium without externally supplied cysteine. These results suggest that a single mutation is responsible for both the Vma<sup>−</sup> phenotypes and the cysteine auxotrophy of the mutant cells.

To determine if the Vma<sup>−</sup> growth phenotype of the vma41 strain is directly related to its cysteine auxotrophy, YOY14-4Ba and cys4Δ cells were supplied with cysteine or reduced glutathione. Under these conditions, the mutant cells were able to grow on minimal medium. Interestingly, externally supplied cysteine was able to partially complement the growth phenotype of YOY14 strain on YEPR medium buffered to pH 7.5 (Fig. 2), with glutathione giving better complementation than cysteine. Conversely, depletion of intracellular glutathione in wild type cells by addition of 50 μM 1-chloro-2,4-dinitrobenzene (57) prevented growth of the cells on YEPR medium buffered to pH 7.5 (data not shown) under conditions where growth of cells on YEPR buffered to pH 5.0 continued. These results indicate that glutathione deficiency results in a pH-dependent (Vma<sup>−</sup>) growth phenotype in yeast.

To confirm that vma41-1 is indeed an allele of CYS4, diploid strain YOY12/pYO38, obtained from a cross between cys4-Δ1 and YOY14-4Ba/pYO38 was sporulated and the resulting tetrads dissected. We found that YOY12 cured of the plasmid pYO38 was unable to sporulate, even after 2 weeks in sporulation medium. This is consistent with previous results indicating that glutathione auxotrophic mutants of Schizosaccharomyces pombe are defective in sporulation (42). Tetrad analysis indicated a 4:0 segregation of the Vma<sup>−</sup> phenotype in YOY12 spores since all Ura<sup>+</sup> spores were Vma<sup>−</sup> and all Ura<sup>−</sup> spores were also Vma<sup>−</sup>. Moreover, when Ura<sup>−</sup> spores lost the plasmid pYO38, they became Vma<sup>−</sup>. Also, YOY12 diploids lacking plasmid pYO38 exhibit a pH-dependent growth phenotype.

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YEPD, pH 7.5, or YEPD, pH 7.5, supplemented with 30 strains of the indicated genotypes were streaked on YEPD, pH 5.0, (Fig. 4 and 27-kDa peripheral subunits of the V-ATPase and the 25-

Western blotting. The results show that the 69-, 60-, 54-, 42-, cys4-

increase the steady state levels of the V-ATPase subunits in

part of a V1 subcomplex, or in the fully assembled V1V0-ATPase. Therefore, we examined the assembly status of the V-ATPase

mutants. Indirect immunofluorescence microscopy, us-

ting antibody recognizes the 60-kDa peripheral subunit by itself, as

part of a V1 subcomplex, or in the fully assembled V1V0-ATPase.

A ring-like staining corresponding to the vacuolar membrane

was detected in the cys4 mutants (data not shown). These results

indicate that the V-ATPase is assembled in cys4 mutants.

These results confirm that vma41-1 is allelic to CYS4.

Characterization of the V-ATPase from cys4 Mutants—All

known vma mutants are unable to accumulate the fluorescent weak base, quinacrine, in their vacuoles as a result of loss of

vacular acidification (25). Quinacrine vital staining was used to

assess vacuolar acidification in YOY14-4Ba (vma41-1) and cys4A mutant cells. Our results (Fig. 3) show that mutants lacking a functional CYS4 gene are unable to accumulate quinacrine in their vacuoles, indicating loss of vacuolar acidification in these mutants. Since GSH was able to complement the pH-dependent growth phenotype of cys4 mutants, it may also be able to restore quinacrine accumulation in the vacuole. Fig. 3 shows partial restoration of vacuolar acidification by GSH as indicated by partial vacuolar staining with quinacrine in the presence of GSH.

To begin to understand the basis of the Vma− growth and vacuolar acidification defects of cys4 mutants, we examined the steady-state levels of several V-ATPase subunits and one specific assembly factor in these cells. Whole cell protein extracts prepared from YOY14-4Ba and cys4A cells were analyzed by Western blotting. The results show that the 69-, 60-, 54-, 42-, and 27-kDa peripheral subunits of the V-ATPase and the 25-
kDa (Vma12p) V-ATPase assembly factor are present in cys4A mutants at normal levels compared with wild type cells (Fig. 4A). Addition of GSH to the growth medium does not increase the steady state levels of the V-ATPase subunits in cysAΔ cells (Fig. 4A). These results indicate that, even in the absence of additional extracellular glutathione, there is sufficient cysteine in the mutants during growth in rich medium to support normal levels of subunit biosynthesis. Previous results (43) have shown that the presence of V-ATPase subunits at normal levels does not always imply assembly of the enzyme. Therefore, we examined the assembly status of the V-ATPase in cys4 mutants. Indirect immunofluorescence microscopy, using antibody against the 60-kDa peripheral subunit of the V-ATPase (13D11) was performed as described (34). The 13D11 antibody recognizes the 60-kDa peripheral subunit by itself, as part of a V1 subcomplex, or in the fully assembled V1V0-ATPase. A ring-like staining corresponding to the vacuolar membrane

was detected in the cys4 mutants (data not shown). These results indicate that the V-ATPase is assembled in cys4 mutants.

Vacuolar acidification was assessed by quinacrine accumulation in the vacuole as described under “Experimental Procedures.” All cells were grown in YEPD, pH 5.0, cysA4 cells + GSH were grown in the presence of 30 μg/ml additional reduced glutathione in the medium. Log phase yeast cells were incubated in 500 μl of phosphate-buffered saline, pH 7, containing 2% glucose and 200 μM quinacrine for 5 min at 30 °C. After staining, cells were washed with 500 μl of phosphate-buffered saline, 2% glucose, and resuspended in 100 μl of the same buffer. Cells were viewed under Nomarski optics to observe normal cell morphology and fluorescence microscopy with a fluorescein isothiocyanate filter to observe vacuolar staining with quinacrine. Each micrograph is a composite of three or four fields.

To address whether the Vma− mutant phenotypes of cys4 mutants are a direct result of the loss (or impairment) of vacuolar ATPase activity, vacuolar membrane vesicles were isolated from wild type and cys4-Δ1 cells and assayed for concanamycin A-sensitive ATPase activity. Concanamycin A is a specific inhibitor of V-ATPases (44). Table II shows that the specific V-ATPase activity of vacuolar membrane vesicles isolated from cys4-Δ1 cells is less than 50% of that of vesicles isolated from the isogenic wild type strain. However, if cys4-Δ1 cells were supplemented with 30 μg/ml GSH during growth, the V-ATPase activity on isolated vesicles is restored to near-wild type levels (Table II). Glutathione supplementation does not have any effect on the V-ATPase activity of vesicles from wild type cells (results not shown). Western analysis of proteins in isolated vacuolar membranes confirm that the reduced level of V-ATPase activity in cys4-Δ1 cells is not a result of reduced amounts of V-ATPase subunits on these membranes. As shown in Fig. 4B, all the subunits monitored are present at wild type levels in the vacuolar membranes of cys4A cells. Addition of GSH to the growth medium does not lead to increased levels of subunits on the membrane. These observations strongly suggest that the cys4 mutation does not affect the assembly or targeting of the vacuolar H+ -ATPase, but has a direct effect on the catalytic activity of the enzyme.

Mutation of Cysteine 261 in the Catalytic Subunit Suppresses Effects of the cys4-Δ1 Mutation—In vitro studies have shown that the clathrin-coated vesicle V-ATPase can be regulated by oxidation and reduction (17). In particular, it was demonstrated that reducing agents activate the enzyme in vitro by promoting disulfide bond interchange between cysteine residues located near the catalytic site (17). Glutathione is the most abundant low molecular weight thiol in the cell, constituting >95% of cellular thiols (45). Most of the glutathione in yeast cells is in the reduced (GSH) form; the thiol-disulfide (i.e. GSH:GSSG) balance is between 50 and 60, similar to other

\[ \text{GSH:GSSG} = 50 \text{ to } 60 \]
eukaryotic cells (46, 47). Since cys4 mutants have been shown to have reduced cellular glutathione concentration (46), and our results (above) indicate that the V-ATPase is expressed at levels similar to wild type in cys4Δ cells, we reasoned that the vma phenotypes of cys4 mutants may result from accumulation of the V-ATPase in an oxidized, inactive form in the less reducing environment resulting from low cellular GSH levels. To test this hypothesis, we used a yeast mutant in which the sulfhydryl-reactive cysteine residue has been mutated. There are three perfectly conserved cysteine residues in the catalytic (A) subunit of all V-ATPases (48). In yeast, these residues are Cys-261, Cys-284, and Cys-538 (numbers without intein); and in bovine they are at positions 254, 277, and 532, respectively. Cys-254 in the bovine enzyme has been identified as the sulfhydryl-reactive residue responsible for inhibition by NEM, and, together with Cys-532 has been implicated in the formation of the inactivating disulfide bond within the A subunit (18). Following this model, mutations that prevent the formation of the proposed disulfide bond are expected to abolish the sensitivity of the V-ATPase to the redox state of the environment. Taiz et al. (48) and Liu et al. (33) have demonstrated that mutation of the cysteine residue at position 261 in yeast to valine abolished the inactivation of the V-ATPase by sulfhydryl reagents and the reversible inactivation in response to oxidizing agents. We constructed a haploid yeast strain (YOY13-2Ca) in which the CYS4 gene has been disrupted and the sole copy of the VMA1 gene (vma1-51) has the critical Cys-261 residue changed to valine by site-directed mutagenesis (Ref. 33, see “Experimental Procedures”). The VMA1 gene product is stably expressed in both the PNY1 strains carrying the C261V mutation in the VMA1 gene and the YOY13-2Ca strain carrying both the C261V mutation and a deletion in the CYS4 gene and the two strains exhibit similar growth on YEPD medium buffered to pH 7.5 (33).

Isolated vacuolar membrane vesicles from YOY13-2Ca and the isogenic PNY1 strains were assayed for V-ATPase activity. Both PNY1 and YOY13-2Ca strains have the same level of V-ATPase activity (Table III) as cells carrying a wild type VMA1 allele. Moreover, in contrast to the cys4Δ1 mutant, the V-ATPase activity of YOY13-2Ca is not increased by the addition of reduced glutathione to the growth medium, indicating that the C261V mutant enzyme, which is no longer sensitive to inhibition by oxidizing agents (33, 48), is no longer influenced by the cys4Δ1 mutation. Western analysis of vacuolar membrane vesicle proteins shows that a number of other V-ATPase subunits are present at normal levels in the PNY1 and YOY13-2Ca strains (results not shown). Our results suggest that the cys4 mutation impairs activity of the yeast vacuolar ATPase by altering the thiol/redox balance in the cell, and that the altered redox balance is sensed (at least in part) by the catalytic subunit of the V-ATPase in vivo.

**Biochemical Properties of the V-ATPase in Vacuoles from cys4Δ Mutants—Analysis of point mutations causing partial defects in V-ATPase activity (49) have indicated that the full pH-dependent growth phenotype requires the loss of >75% of V-ATPase activity on the vacuole. However, cys4 mutants exhibit full Vma− phenotypes but have approximately 47% wild**

### Table III

| Strain | Specific activity | Wild type activity |
|--------|------------------|--------------------|
|        | μmol/min/mg protein | %                  |
| NO11–2 (WT) | 0.79 ± 0.10 (4) | 100                |
| PNY1   | 0.82 ± 0.07 (4)  | 104                |
| YOY13–2Ca | 0.80 ± 0.07 (4) | 101                |
| YOY13–2Ca + GSH | 0.79 ± 0.13 (4) | 100                |
Preparation of vacuolar membrane vesicles and determination of concomitant A-sensitive ATPase activity are as described in the legend to Table II. Initial incubations were performed with 5 μg of vesicle protein in a total of 100 μl of incubation buffer (15 mM MES-Tris, pH 7.0, 4.8% glycerol) and incubation mixtures were added to a 1 ml of coupled enzyme assay mixture. The specific activities of untreated vesicles were 3.4 and 1.6 μmol of ATP/min of protein for wild type and cys4-D1, respectively. For NEM, then DTT samples, vesicles were treated with 100 μM NEM for 10 min followed by 100 mM DTT for 30 min. For H2O2, then DTT samples, vesicles were treated with 100 mM H2O2 for 10 min followed by 100 mM DTT for 30 min. For NaIO3, then DTT samples, vesicles were treated with 10 mM NaIO3 for 5 min followed by 100 mM DTT for 30 min.

### Table IV

| Treatment                  | % ATPase activity remaining |
|----------------------------|----------------------------|
| Wild type                  |                           |
| 9 h at 23 °C               | 100 ± 2.5 (3)              |
| 10 mM GSH, 2 h at 23 °C    | 75.8 ± 7.4 (4)             |
| 100 mM DTT, 30 min on ice | 70.1 ± 7.3 (2)             |
| NEM, then DTT              | 100 ± 1.0 (2)              |
| 10 mM H2O2, 10 min at 23 °C| 26.0 ± 7.3 (2)             |
| H2O2, then DTT             | 57.5 ± 5.5 (2)             |
| 10 mM NaIO3, 10 min at 23 °C| 21.0 ± 5.5 (2)             |
| NaIO3, then DTT            | 60.0 ± 10.0 (2)            |

The enzymatic behavior of the residual V-ATPase activity in vacuolar vesicles from S. cerevisiae wild type or cys4-D1 mutant cells (data not shown). This result does not prove that the yeast V-ATPase is not subject to activation during purification; further experiments and/or optimization of the conditions may be needed to demonstrate this. To further investigate the enzymatic properties of the V-ATPase in vacuolar membranes from cys4-D1 mutants grown in the absence of added extracellular glutathione, we examined the effects of several chemical agents that have been linked to oxidative inhibition of V-ATPase activity in vitro in other systems (16, 23) on the yeast enzyme. Our results (Table IV) show that the enzymatic behavior of the residual V-ATPase activity from cys4-D1 vacuolar vesicles is similar to that of the wild type enzyme. The “residual” ATPase activity represents the activity in vacuoles isolated from cys4-D1 mutants grown in the absence of additional glutathione. V-ATPase activity is stable at 0 °C or room temperature (23 °C) for up to 9 h by warming the incubation after 5 h at room temperature (23 °C) for 3 h (data not shown) in the absence and presence of glucose (Table IV). The sensitivity of V-ATPase activity from cys4-D1 membranes to NEM and concanamycin A, two specific inhibitors of V-ATPases, is identical to that of the wild type enzyme (Table IV and data not shown). Previous evidence indicates that NEM inhibits V-ATPase activity by modification of Cys-261 of the catalytic subunit (16, 33, 48). Therefore, the inhibition of the V-ATPase in cys4-D1 membranes suggests Cys-261 is available for NEM binding in the population of enzyme responsible for the residual ATPase activity. Oxidizing agents (iodate and hydrogen peroxide) inhibit the ATPase activity of both enzymes at similar concentrations. Enzyme activity is partially restored by treatment with DTT after iodate or peroxide treatment (Table IV). DTT alone activates ATPase activity up to 30%. In contrast to previous results which show that GSH activates the N. crassa V-ATPase (23), addition of GSH at concentrations ranging from 500 μM to 30 mM did not activate the yeast V-ATPase in vitro (Table III and data not shown). In fact, our in vitro experiments show that GSH reproducibly inactivates the yeast V-ATPase in a concentration-dependent manner. Notably, treatment of isolated vesicles with GSH did not restore wild type V-ATPase activity to the enzyme isolated from cys4-D1 mutant (Table IV), indicating that intracellular GSH (manipulated by extracellular GSH concentrations) may not act directly on the V-ATPase to restore activity in the cys4 mutants.

To determine if the cys4-D1 mutation causes any structural defects in the V-ATPase, we purified the V-ATPase from vacuolar membranes isolated from wild type and cys4-D1 cells by glycerol gradient centrifugation as described (50, 51). Our results suggest that the V-ATPase from cys4 mutant cells may be structurally different from the V-ATPase from wild type cells. First, in cys4-D1 vacuolar membranes, 39% of the specific V-ATPase activity is lost upon detergent solubilization, a treatment that slightly increases the specific activity of the enzyme from wild type membranes. Second, the residual V-ATPase activity of solubilized cys4-D1 vacuolar membranes is completely lost after glycerol gradient centrifugation. No activity is detected in any of the fractions collected from cys4-D1 membranes (data not shown) but solubilized vacuolar membranes from wild type cells give a sharp peak of V-ATPase activity centered around fraction 9. These data indicate that even though vacuolar vesicles from cys4-D1 cells contain a considerable V-ATPase activity, this activity is significantly less stable than that of wild type vesicles. To check whether the observed loss of V-ATPase activity upon glycerol gradient centrifugation is a result of dissociation of the V-ATPase into partial complexes or individual subunits, proteins in fractions obtained from glycerol gradient were precipitated, separated by SDS-PAGE, and analyzed by silver staining. Fig. 5 shows that the bulk of the assembled V-ATPase in solubilized vacuolar membranes from wild type (Fig. 5A) and cys4-D1 (Fig. 5B) cells fractionates to similar portion of the gradient. Previous results (58) have shown that in yeast mutants lacking a functional copy of the VMA13 gene product, the V-ATPase is assembled but inactive. Fraction 9, which contains the peak of V-ATPase activity in wild type membranes was analyzed by Western blotting, using polyclonal antisera against the 54-kDa Vma13p and monoclonal antibody against the 69-kDa Vma13p. Fig. 5C shows that Vma13p and Vma1p are present at wild type levels in the V-ATPase purified from cys4-D1 cells. These results indicate that the loss of activity in the V-ATPase purified from cys4-D1 cells is not due to loss of the 54-kDa Vma13p. Western blots of the 69-, 60-, 42-, and 27-kDa V1 subunits and the 100-kDa V0 subunit (data not shown) confirmed that fraction 9, which contains the peak of V-ATPase activity in wild type membranes, also represents a single peak of V1 subunits. V1 subunits peak at both fractions 9 and 12–13, consistent with previous results (35). The solubilized cys4-D1 membranes show a very similar pattern, indicating that the V-ATPase subunits are present at comparable levels in the purified V-ATPase from cys4-D1 and wild type cells, and there do not appear to be multiple populations of partially assembled enzyme in the cys4-D1 vacuolar membranes.
Complementation analysis revealed that VMA41 was able to restore the cysteine-independent growth to a yeast strain in which the CYS4 gene has been disrupted (39), indicating that Cys4p activity is conserved in evolution. Cys4p is involved in cysteine biosynthesis through the transulfuration pathway in S. cerevisiae where it catalyzes the first of two reactions in the biosynthesis of cysteine from homocysteine (40, 53). Loss of Cys4p activity confers a cysteine dependence which cannot be relieved by supplementation with other organic sulfur compounds, such as methionine (40), but is effectively relieved by glutathione (41). The observation that reduced glutathione as an exogenous source of sulfur is better than cysteine has been attributed to the poor uptake (40) and toxicity (45) of cysteine. In addition to protein synthesis, cysteine is required for glutathione synthesis; therefore, because cys4 mutants are unable to synthesize cysteine, they will be deficient in glutathione synthesis. In agreement with this, cys4 mutants have been shown to have greatly reduced cellular GSH (13.6% of wild type; Ref. 46). Since GSH represents more than 95% of the non-protein thiol pool in wild type yeast cells (47), GSH-deficient strains should have a much less reducing cytosol than wild type cells.

Glutathione is the most abundant low molecular weight peptide and the most prevalent cellular thiol (45). GSH functions in metabolism, transport, cellular protection, and catalysis, particularly in the reduction of the disulfide linkages of proteins and other molecules, and plays an essential role in cell growth (42, 45). The ability of yeast cys4 mutants to grow in YEPD (which contains 1% yeast extract) buffered at pH 5.0 is consistent with the fact that the yeast extract in the medium contains glutathione (42). Our results showing that cys4-Δ cells grown in YEPD, pH 5.0, have reduced levels of V-ATPase activity on isolated vacuolar membranes despite the presence of some glutathione in the medium can be explained by reports on S. pombe, indicating that glutathione synthesis is more efficient than its uptake (42). In these studies, despite the presence of ~0.25 mM glutathione in the medium, the GSH level in gcs1Δ cells (equivalent of S. cerevisiae gsh1Δ mutants, which lack one enzyme involved in glutathione biosynthesis; Ref. 42) was only 20% that of wild type cells. It is possible that uptake from the medium provides enough cysteine to allow synthesis of normal levels of V-ATPase subunits, but does not provide sufficient GSH to fully overcome the inhibition of the V-ATPase in cys4-Δ cells. Further addition of GSH to the medium may stimulate uptake by a concentration effect to the point where the intracellular GSH level is enough for partial activation of the V-ATPase. Chaudhuri et al. (42) have reported that supplementation of GSH− mutants of S. pombe with reduced glutathione (up to 0.25 mM) restores a Gsh− phenotype but not pigmentation in adenine-limiting medium. The emerging theme from studies of GSH metabolism is that there is a minimum critical level of GSH required for growth and protein synthesis which is lower than that required for the other functions of GSH in metabolism, detoxification, and transport (47).

**Effect of Loss of Cys4p Activity on Cellular Thiol Balance**—The yeast CYS4 gene was identified as the gene responsible for cystathionine-β-synthase activity (40, 41, 52). The human homolog of CYS4 (CBS) is able to restore cysteine-independent growth to a yeast strain in which the CYS4 gene has been disrupted (39), indicating that Cys4p activity is conserved in evolution. Cys4p is involved in cysteine biosynthesis through the transulfuration pathway in S. cerevisiae where it catalyzes the first of two reactions in the biosynthesis of cysteine from homocysteine (40, 53). Loss of Cys4p activity confers a cysteine dependence which cannot be relieved by supplementation with other organic sulfur compounds, such as methionine (40), but is effectively relieved by glutathione (41). The observation that reduced glutathione as an exogenous source of sulfur is better than cysteine has been attributed to the poor uptake (40) and toxicity (45) of cysteine. In addition to protein synthesis, cysteine is required for glutathione synthesis; therefore, because cys4 mutants are unable to synthesize cysteine, they will be deficient in glutathione synthesis. In agreement with this, cys4 mutants have been shown to have greatly reduced cellular GSH (13.6% of wild type; Ref. 46). Since GSH represents more than 95% of the non-protein thiol pool in wild type yeast cells (47), GSH-deficient strains should have a much less reducing cytosol than wild type cells.

Finally, the V-ATPase was purified from the YOY13-2Ca strain. Western analysis and silver staining of SDS-PAGE gels indicate that the polypeptide composition of the V-ATPase purified from YOY13-2Ca vacuolar membranes is indistinguishable from that of the enzyme purified from an isogenic wild type strain (data not shown). However, the specific ATPase activity of the V-ATPase purified from YOY13-2Ca cells was 5.2 μmol/min/mg compared with 8.0 μmol/min/mg for the enzyme from the isogenic wild type strain.

**DISCUSSION**

In a genetic screen designed to identify novel genes affecting the function of the yeast vacuolar H+-ATPase, we obtained a yeast strain YOY14-4Ba containing the yma41-1 mutation. Complementation analysis revealed that VMA41 is allelic to CYS4. Our results indicate that although the CYS4 gene product is not essential for assembly of the V-ATPase, it is required for full enzyme activity. These data provide the first in vivo evidence for the regulation of vacuolar H+-ATPase activity by the redox state of its environment.

**Effects of Loss of Cys4p Activity on V-ATPase Activity**—We show here that cys4 mutants exhibit growth phenotypes characteristic of the loss of V-ATPase activity in vivo. In addition to the cysteine-dependent growth phenotype characteristic of cys4 mutants (40, 41), the yma41-1 and cys4-Δ1 mutants display Vma− growth phenotypes (Fig. 2 and data not shown) and are unable to accumulate quinacrine in their vacuoles (Fig. 3), indicating loss of vacuolar acidification. Isolated vacuolar ves-
icles appear to contain fully assembled V-ATPase complexes, but have reduced V-ATPase activity. Significantly, the \textit{uma} growth and acidification defects of the \textit{cys4} mutants can be suppressed by addition of GSH to the growth medium, and vacuoles isolated from \textit{cys4-\Delta1} strain after growth in the presence of extracellular GSH have wild type levels of V-ATPase activity.

Vacuolar ATPases have been shown to be sensitive to the redox state of their environment \textit{in vitro} (16–18, 23, 33). The effects of the \textit{cys4-\Delta1} mutant on the yeast V-ATPase activity can be suppressed by the previously characterized \textit{uma1–51} mutation (33, 48), in which the highly conserved cysteine 261, which is predicted to be at the catalytic site of the yeast 69-kDa subunit (Vma1p), has been replaced by valine (Table III). The \textit{uma1–51} mutation has previously been demonstrated to render the yeast V-ATPase insensitive to oxidative inactivation \textit{in vitro}, presumably by preventing formation of an inactivating disulfide bond involving cysteine 261 of the catalytic subunit (33). This result indicates that the functional and structural defects of the V-ATPase in \textit{cys4} mutants may be derived from oxidative inactivation involving disulfide bond formation in the catalytic subunit of the enzyme \textit{in vivo} as a result of reduced levels of cytosolic GSH. Although enzymatic characterization of the V-ATPase from \textit{cys4-\Delta1} vacuolar membranes indicated that the enzyme could not be readily reactivated by reducing agents \textit{in vitro} (Table IV), this result may not be surprising, because even after a brief oxidative inactivation, dependent on cysteine 261, of the yeast V-ATPase \textit{in vitro}, ATPase activity could only be partially restored by reducing agents (33). If, as proposed, reactivation of the enzyme after oxidative inactivation requires disulfide interchange in the enzyme (18), this interchange may well be more difficult to induce than a simple sulfhydryl reduction.

There is evidence, however, that the effects of the \textit{cys4} mutations on the V-ATPases may not be mediated solely through disulfide bond formation by the catalytic site cysteine 261 \textit{in vivo}. Vacuolar membranes isolated from \textit{cys4-\Delta1} mutants grown in the absence of added extracellular glutathione contain a residual V-ATPase activity (47% of wild-type) that is sensitive to NEM, suggesting that cysteine 261 is not disulfide-bonded in the population of enzyme responsible for this activity. This residual activity could be derived from spontaneous activation of a population of the V-ATPase during vacuolar isolation, as reported by Feng and Forgac (17) for the bovine clathrin-coated vesicle enzyme. The residual activity found in the yeast \textit{cys4-\Delta1} membranes proved to be unstable to detergent solubilization and glycerol gradient purification, however, indicating that the enzyme complexes responsible for the activity are functionally defective, even though we detected no obvious structural defect in the isolated enzyme. Furthermore, previous results have shown that the onset of the full pH-dependent growth phenotype requires the loss of more than 75% of V-ATPase activity at the vacuole (49). The fact that \textit{cys4} mutants exhibit the full range of \textit{Vma} phenotypes, including pH-dependent growth and total loss of quinacrine accumulation, strongly suggests that the residual activity in \textit{cys4-\Delta1} vacuoles is not functional \textit{in vivo}. The fact that the specific activity of the V-ATPase purified from the \textit{yo13-2C}a strain is less than that of the comparable wild type strain is additional evidence for an effect of the \textit{cys4} mutation not mediated through disulfide bond formation involving cysteine 261 at the catalytic site.

Taken together, these results indicate that the loss of V-ATPase activity in \textit{cys4} mutants is the result of two separate effects of the mutation on the V-ATPase. The ability of the \textit{uma1–51} mutation to suppress the V-ATPase activity defect indicates that one of these effects is oxidation of cysteine 261 in the catalytic subunit. This oxidative inactivation appears to be reversed or suppressed by addition of excess extracellular GSH to yield an enzyme capable of function \textit{in vitro}, based on restoration of wild type levels of ATPase activity at the vacuole, and \textit{in vivo}, based on the partial restoration of quinacrine staining shown in Fig. 3. A second population of enzyme is responsible for the 47% residual activity of isolated \textit{cys4-\Delta1} membranes. This enzyme population exhibits a structural defect that has not been fully defined but does not appear to involve cysteine 261. It is unclear whether this enzyme population is ever functional \textit{in vivo}. We detected no obvious structural defect in the V-ATPase purified from \textit{cys4} strains, but due to the size of the V-ATPase (at least 750-kDa), we might not detect small differences in subunit composition between the V-ATPase from wild type and \textit{cys4} membranes. Therefore, it is possible that the loss of V-ATPase activity may be due to the absence of some unidentified subunit(s) of the V-ATPase as a result of oxidative inhibition \textit{in vivo}.

\textbf{Oxidation and Reduction as a Means of Enzyme Regulation}—Several mechanisms have been proposed for the regulation of vacuolar H\textsuperscript+-ATPases (reviewed in Ref. 12). These include disassembly and reassembly of peripheral \textit{V}_{1} integral \textit{V}_{0} domains, recruitment of enzyme in vesicles to the site of action, regulation of parallel ion channels that compensate for the electrogenicity of the V-ATPase, changes in the degree of coupling between ATP hydrolysis and proton pumping, and reversible disulfide-bond interchange between conserved cysteine residues near the catalytic site.

The occurrence of reactive sulfhydryl groups is a common feature of enzymes that are inactivated by oxidizing agents such as iodoacetamide or N-ethylmaleimide and are stabilized or activated by reducing agents such as dithiothreitol. Oxidative inhibition \textit{in vitro} has been demonstrated for several enzymes including the V-ATPase of bovine clathrin-coated vesicles (16), the \textit{N. crassa} V-ATPase (22), and the rabbit muscle phosphofructokinase (54). In a recent study, it was shown that the loss of intracellular GSH causes a reversible impairment of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter of Madin-Darby canine kidney cells (55). This type of result has led to the suggestion that modulation of the thiol/disulfide ratio \textit{in vivo} may serve as a “third messenger” in response to signals such as cAMP levels (54).

Our findings provide the first \textit{in vivo} evidence for the regulation of a V-ATPase by thiol/disulfide exchange, but do not necessarily address how important this regulation mechanism may be for the activity of the yeast V-ATPase \textit{in vivo} in wild type cells. The oxidative stress imposed by the \textit{cys4} mutations, which revealed the evidence of oxidative inactivation of the yeast V-ATPase \textit{in vivo}, probably exceeds the stresses encountered by a wild type cell under normal growth conditions. Nevertheless, a variety of biochemical studies, combined with the conservation of the catalytic site cysteine corresponding to Cys-261 of the yeast Vma1p, indicate that oxidative inactivation is a general characteristic of V-ATPases. A number of other eukaryotic cells possessing high levels of V-ATPases may encounter conditions of severe oxidative stress regularly (for example, macrophages undergoing an oxidative burst), and our results suggest that oxidative inactivation of the V-ATPase could be a very important mechanism for regulation of V-ATPase activity in these cells.

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