Loss of Vacuolar Proton-translocating ATPase Activity in Yeast Results in Chronic Oxidative Stress

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Yeast mutants lacking vacuolar proton-translocating ATPase (V-ATPase) subunits (vma mutants) were sensitive to several different oxidants in a recent genomic screen (Thorpe, G. W., Fong, C. S., Alic, N., Higgins, V. J., and Dawes, I. W. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6564–6569). We confirmed that mutants lacking a V₁ subunit (vmaΔ), Vₒ subunit, or either of the two Vₒ subunit isoforms are acutely sensitive to H₂O₂ and more sensitive to menadione and diamide than wild-type cells. The vmaΔ mutant contains elevated levels of reactive oxygen species and high levels of oxidative protein damage even in the absence of an applied oxidant, suggesting an endogenous source of oxidative stress. vmaΔ mutants lacking mitochondrial DNA showed neither improved growth nor decreased sensitivity to peroxide, excluding respiration as the major source of the endogenous reactive oxygen species in the mutant. Double mutants lacking both Vₒ and components of the major cytosolic defense systems exhibited synthetic sensitivity to H₂O₂. Microarray analysis comparing wild-type and vmaΔ mutant cells grown at pH 5, permissive conditions for the vmaΔ mutant, indicated high level up-regulation of several iron uptake and metabolism genes that are part of the Aft1/Aft2 regulon. TSA2, which encodes an isofrom of the cytosolic thioredoxin peroxidase, was strongly induced, but other oxidative stress defense systems were not induced. The results indicate that V-ATPase activity helps to protect cells from endogenous oxidative stress.

Both prokaryotic and eukaryotic cells have multiple mechanisms for preventing and counteracting the effects of oxidative stress (1). Reactive oxygen species (ROS) are normal products of aerobic metabolism, but excess ROS accumulation due to perturbation of control mechanisms can result in accumulation of mutations, premature aging, and cell death (2–4). Many of the central mechanisms for controlling redox balance in the cell are highly conserved. These mechanisms include 1) proteins involved directly in regulating redox balance or repairing effects of oxidative stress, including the well known components of the glutathione- and thioredoxin-dependent reduction systems, superoxide dismutase, catalase, and the pentose phosphate pathway, and 2) a transcriptional response centered around recruitment of the “redox-specific” transcription factors to promoters of these and other antioxidant response genes (2, 3).

Only recently has the full spectrum of genes important for control of oxidative stress begun to be appreciated, through microarray analysis of responses to a variety of oxidants and screens of ordered S. cerevisiae deletion mutant arrays (5–7). Genomic screens have revealed that deletions in many genes not previously associated with redox balance result in sensitivity to one or more applied oxidants (6–8). These screens also revealed that response to oxidative stress may be more specific than previously appreciated, since many deletion mutants show sensitivity to one type of oxidant but little sensitivity to others (7, 8). These results suggest that the cell tailors its response to different stresses rather than having a single general response pathway (9). However, certain deletion strains do show sensitivity to multiple different applied oxidants (7, 8). This may indicate that the deletions in these genes affect protective mechanisms that are used in multiple contexts and/or that the deletions result in a chronic state of oxidative stress that render the mutants unable to cope with further stress. Consistent with this, mutants lacking important antioxidant functions also show evidence of oxidative stress or damage even in the absence of an applied oxidative stress (10, 11). In these cases, the screens may be revealing previously unappreciated sources of oxidative stress and the cellular mechanisms that contain them.

Yeast mutants lacking subunits of the vacuolar proton-translocating ATPase (V-ATPase) have been identified in multiple genomic screens for sensitivity to different forms of oxidative stress (6–8). The V-ATPase is a highly conserved proton pump responsible for acidification of organelles such as the lysosome/vacuole, Golgi apparatus, and endosomes in all eukaryotic cells (12, 13). V-ATPases have also been recruited to a variety of more specialized functions, often involving high level expression of the enzyme at the plasma membrane of polarized cells, in addition to intracellular compartments (13, 14). The yeast S. cerevisiae does not appear to express a plasma membrane form of the V-ATPase and has proven to be an excellent model for the “constitutive” functions of V-ATPases associated with acid-
Yeast V-ATPase Protects against Oxidative Stress

All eukaryotic V-ATPases are multisubunit enzymes comprised of a complex of peripheral membrane proteins, V₁, attached to a complex of integral membrane proteins, Vₒ. Deletion of any V-ATPase subunit gene, except VPH1 and STV1, which encode organelle-specific isoforms of one of the Vₒ subunits, results in a common Vma⁻ phenotype, characterized by a pH-dependent conditional lethality and sensitivity to elevated extracellular calcium concentrations (12). In addition, vma mutants show a variety of other defects that are not completely understood, including poor growth on nonfermentable carbon sources, hypersensitivity to multiple drugs, and increased sensitivity to transition metals, such as iron, copper, and zinc (15–17).

In this work, we confirm that vma mutants are highly sensitive to oxidative stress, particularly H₂O₂, and further explore the basis of this sensitivity. We find that the vma mutants show evidence of chronic oxidative stress, including elevated ROS levels and protein modifications characteristic of accumulated oxidative damage, even in the absence of any added oxidant, but the electron transport chain is not the major source of the stress in these cells. vma mutations result in a synthetic sensitivity to peroxide when combined with mutations in many of the components of the cytoplasmic machinery for redox control, including the Cu/Zn-superoxide dismutase, glutathione reductase, glutathione synthetase, thioredoxins, thioredoxin peroxidases, and components of the oxidative arm of the pentose phosphate pathway, suggesting that vacuolar acidification is not simply involved in supporting one of these mechanisms central to redox balance. Microarray analysis under conditions permissive for growth of the vma2 mutant (pH 5) indicates high level up-regulation of the cytosolic thioredoxin peroxidase Tsa2p and induction of the Aft1p/Aft2p regulon in the vma2 mutant. Based on these results, we suggest several possible explanations for the sensitivity of vma mutants to applied oxidative stresses, such as hydrogen peroxide.

**EXPERIMENTAL PROCEDURES**

Materials and Strains—Hydrogen peroxide was obtained from Acros. Menadione and diamide were obtained from Sigma. Dihydrorhodamine 123 was obtained from Sigma, and the OxyBlot kit was purchased from Chemicon International. Yeast extract/peptone/dextrose (YPED) and synthetic complete (SC) were prepared as described (18).

All strains were obtained from the Open Biosystems or Euro- fand haploid deletion collections and were based on BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0Δ). All strains in the collection have a complete replacement of the open reading frame with the kanMX marker in the BY4741 background (19). The BY4742 vma2Δ::Nat’ strain was constructed as described (20). Double mutant spores were isolated by mating the indicated strain from the deletion collection with the BY4742 vma2Δ strain, selecting diploids on YEPD containing 200 µg/ml kanamycin and 200 µg/ml clonNat (Werner Biolabs) and then sporulating the resulting diploids and dissec teting tetrads on YEPD plates buffered to pH 5. Haploid spores resistant to both kanamycin and clonNat were identified.

The wild-type BY4741 and the BY4741 vma2Δ strain were converted to rhlΔ strains by extended incubation with 25 µg/ml ethidium bromide as described (21). Loss of mitochondrial DNA was confirmed by 4’,6-diamidino-2-phenylindole staining.

For dilution assays to test peroxide sensitivity, hydrogen peroxide was added to SC to give the final concentration indicated. Plates were used within 2 days of the assay. Liquid cultures of strains to be tested were grown to log phase, adjusted to identical density, and then serially diluted (10-fold dilutions) before spotting on the plates.

Genetic and Biochemical Tests for Oxidative Stress Sensitivity and Oxidative Damage—Dihydrorhodamine 123 (DHR) staining was performed as described by Madeo et al. (23). Briefly, DHR was dissolved in ethanol to make a 2.5-mg/ml stock.Liquid cultures of BY4741 and BY4741 vma2Δ strains and the corresponding rhlΔ cells were grown overnight in SC, and DHR was added to a final concentration of 5 µg/ml. Cells were viewed using a Zeiss Axioskop 2 fluorescence microscope and a rhodamine filter set after 4.5 h of staining. Images were captured using a Hamamatsu CCD camera. For quantitation of the percentage of cells stained with the dye, 280–350 cells from each strain were scored in two independent experiments. Samples were compared using the two-sample t test assuming equal variances.

Carbonylated proteins were detected by reaction with 2,4-dinitrophenyl (DNP) hydrazine and detection of DNP-modified protein by Western blotting with rabbit anti-DNP polyclonal antibody according to protocols provided with the OxyBlot kit. Yeast cells were prepared as described (24) with the following modifications. 5 A₆₀₀ units of each strain were isolated during log phase growth, converted to spheroplasts, and lysed by the addition of 0.5 ml of cold 0.6 m sorbitol, 1 mM EDTA, 50 mM dithiothreitol, pH 7.0, and vortex mixing for 2 min. Unlysed cells were pelleted, and the supernatant was used for derivatization. A mock derivatization, in which all reagents except DNP-hydrazine were added to the supernatant, was carried out in parallel for each strain. After neutralization of the reaction, equal amounts of protein for each sample were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

The GFP-YAP1 plasmid was a generous gift from Scott Moje-Rowley (25). The plasmid was transformed into wild-type and vma2Δ strains, and transformants were selected and maintained on fully supplemented minimal medium lacking uracil. Localization of Yap1p was visualized after overnight growth of the cells in SC-uracil.
Yeast V-ATPase Protects against Oxidative Stress

Microarray and Northern Analyses—Total yeast RNA was isolated from wild-type and vma2Δ mutant yeast cells grown to early log phase in YEPD, pH 5, as described (26). The total RNA obtained was then further purified using a Qiagen RNeasy RNA purification kit, and then the quality and quantity of the RNA was analyzed using an Agilent RNA nanochip on an Agilent 2100 Bioanalyzer. Duplicate RNA samples (from two independent isolations from the vma2Δ strain and a single isolation from the wild-type strain) were subjected to microarray analysis using Affymetrix Yeast Gen2 chips and standard eukaryotic one-cycle amplification and labeling procedures. Arrays were scanned on a GeneChip 3000 scanner (Affymetrix) and analyzed by GeneChip Operating System software (Affymetrix) to generate present and absent calls and assess sample quality. Subsequently, the .cel format image files were imported into GeneSpring GX (Agilent) and normalized using the robust multiaarray average method (27). For this report, we determined the fraction of genes that were increased or decreased in the mutant versus wild type strains at the two-tailed 99th percentile confidence level (defined as ±2.58 S.D. values from the mean expression ratio comparing the two strains). In the present study, this translated to cut-off values of 2.1-fold up or down in assessing changes in expression in the mutant strains (see supplemental Fig. 1).

For Northern analysis, two independent RNA samples were prepared from both the wild type and vma2Δ mutant as described above. RNA was separated on a 2.2 m formaldehyde denaturing gel, transferred by capillary action to Duralon membrane, and fixed to the blot by UV cross-linking (260 nm for 1 min), as described by Sambrook and Russell (28). Probes against the indicated genes were prepared by PCR amplification of 250–400-bp regions of the open reading frames of the genes, followed by labeling with [α-32P]dCTP using a Stratagene Prime-It random primer labeling kit. Blots were hybridized with the probes as described (28) and then washed and analyzed on an Amersham Biosciences Typhoon PhosphorImager.

RESULTS

Yeast vma Mutants Are Acutely Sensitive to Oxidative Stress—In order to confirm that the vma mutants were sensitive to external oxidative stress, we challenged two of the vma deletions, vma2Δ and vma3Δ, with several concentrations of H₂O₂. These two mutations affect the two different sectors of the V-ATPase; vma2Δ lacks the B subunit of the V₀ sector but contains assembled V₁ sectors at the vacuole, and vma3Δ lacks the c subunit of the V₀ sector and contains assembled V₁ sectors in the cytosol (29). Fig. 1A shows a halo assay for wild type, vma2Δ, and vma3Δ in which varied H₂O₂ concentrations were applied to filter disks on top of a fresh layer of cells, and the size of the area of inhibited growth around the disk provides a measure of sensitivity (a larger halo indicates sensitivity to a lower concentration of H₂O₂ as it diffuses from the filter). It is clear from Fig. 1A that both the vma2Δ and vma3Δ mutants exhibit much larger halos than the wild-type control strain at every peroxide concentration tested, suggesting that the mutant strains are very sensitive to peroxide stress. In Fig. 1B, the sensitivity of the vma2Δ strain to two other oxidants, diamide and menadione, was compared with the wild-type strain.

The vma2Δ mutant is only slightly more sensitive to diamide than the wild-type strain at pH 5, but there is an increase in its relative sensitivity at pH 7. In contrast, H₂O₂ (at the concentrations used in Fig. 1A) almost completely inhibits growth of vma2Δ and vma3Δ cells at neutral pH (data not shown). Menadione sensitivity could only be measured at elevated pH and was clearly more pronounced in the vma2Δ mutant than in wild type. The sensitivity of the vma3Δ mutant to these oxidants was similar to or slightly greater than that of the vma2Δ mutant (data not shown). These data support previous indications that vma mutants are sensitive to various forms of applied oxidative stress but also suggest that the mutants are exquisitely sensitive to H₂O₂.

Both the vma2Δ and vma3Δ mutations disrupt subunits that are required for all V-ATPase activity in the cell (12). One subunit of the V-ATPase, the V₀ a subunit, is present as two organelle-specific isoforms, Vph1p and Stv1p, which have steady state localizations at the vacuole and Golgi apparatus/endosomes, respectively, in wild-type cells (30, 31). Fig. 1C shows that both vph1Δ and stv1Δ mutants are much more sensitive to peroxide at pH 5 than wild-type cells, although neither is as sensitive as the vma2Δ or vma3Δ mutants. These results suggest that both vacuolar Vph1p-containing V-ATPases and the Golgi/endosome Stv1p containing V-ATPases contribute to resistance to oxidative stress.

As a means of quantitating the relative strength of the sensitivity of the vma mutants to peroxide stress, we compared the relative halo size of the vma2Δ challenged with 2 and 5% peroxide to those of deletions of the two superoxide dismutases (Sod1p and Sod2p) in yeast, which are firmly established as important defenses against oxidative stress (32). As shown in Fig. 1D, vma2Δ mutants have significantly larger halos than wild-type cells challenged with 2 and 5% peroxide, consistent with the data shown in Fig. 1A. Furthermore, the halo sizes for the vma2Δ mutant are comparable with those for either superoxide dismutase mutant, suggesting that loss of V-ATPase activity compromises the resistance of cells to peroxide to a comparable degree.

vma Mutants Are under Chronic Oxidative Stress—Cells that are highly sensitive to an applied oxidative stress often contain elevated levels of reactive oxygen species (ROS), which can be present in a number of different chemical forms (33), even in the absence of an applied stress. An increase in steady-state ROS levels can be detected with redox-sensitive dyes. In order to compare ROS levels in the wild-type and vma mutants, we incubated the cells with DHR. DHR enters the cell but is non-fluorescent unless it is oxidized by intracellular oxidants to form rhodamine 123. Wild-type and vma2Δ mutants were grown overnight in fully supplemented minimal medium, and then DHR was added for an additional 4.5-h incubation. Fig. 2 shows DHR staining of one field of wild-type and of a vma2Δ mutant to these oxidants was similar to or slightly greater than that of the vma2Δ mutant (data not shown). These data support previous indications that vma mutants are sensitive to various forms of applied oxidative stress but also suggest that the mutants are exquisitely sensitive to H₂O₂.

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**FIGURE 1.** Sensitivity of V-ATPase deletion mutants to various forms of oxidative stress. A, halo assays comparing sensitivity of wild-type BY4741 cells (wt), BY4741 vma2Δ, and BY4741 vma3Δ cells to H2O2. Cells grown to logarithmic phase in YEPD buffered to pH 5 were applied to a YEPD, pH 5, plate as an overlay. Filter paper disks were placed on top of the overlay, and 1, 2, 5, and 10% H2O2 was applied to the four disks shown. The plates were grown for 2 days at 30 °C. Clear or lightly cloudy areas surrounding each disk represent inhibited cell growth due to H2O2 diffusion from the disk, and the size of these “halos” is dependent on the sensitivity of the strain to H2O2 (22). There was no growth inhibition around a control disk to which no H2O2 was applied (not shown). B, halo assays comparing the sensitivity of wild-type and vma2Δ cells to diamide and menadione. The assay was prepared as in A, except that 250 mM (left) and 500 mM (right) diamide or 20 mM (left) and 40 mM (right) menadione were applied to the disks. For the middle set of panels, the plate supporting the overlay was YEPD, pH 7, rather than YEPD, pH 5. C, halo assays comparing the sensitivity of BY4741 vma2Δ and BY4741 stv1Δ to H2O2. Disks on the right were spotted with 10% H2O2, and those on the left were spotted with 5% H2O2. The assays were done as described in A, D, comparison on relative halo sizes for different mutants in response to 5 and 2% H2O2. Halo assays were performed on the indicated strains as described for A, and the size of the halo surrounding the 5% H2O2 (light gray) and 2% H2O2 (dark gray) filters were measured for the mutant and wild-type strains. Relative halo size was determined by dividing the size of the mutant halo by the size of the wild-type halo determined in parallel. Bars represent the mean ± S.E. for eight independent experiments for vma2Δ and two independent experiments for sod1Δ and sod2Δ.

Cells that are under chronic oxidative stress accumulate protein modifications of several different types (34, 35). One of these modifications is protein carbonylation, and levels of carbonylated protein have frequently been used as a measure of chronic oxidative stress (24, 35). We detected protein carbonylation using the OxyBlot system, in which carbonylated proteins are further derivatized with DNP, which can then be detected by Western blotting. As shown in Fig. 2B, lysates from wild-type cells contain very little DNP-reactive carbonylated protein, but the vma2Δ mutant contains high levels of protein carbonylation, even though no extracellular oxidant was added in these experiments.

**What Is the Source of ROS Accumulation in the vma Mutants?—**Reactive oxygen species can arise from a number of different sources and processes, but mitochondria, and specifically the respiratory chain, are frequently one of the major sources (36). In yeast, at least one subunit of each complex of the electron transport chain is encoded by mitochondrial DNA, and the cells can survive by fermentative growth in the absence of mitochondrial DNA and respiratory function. Therefore, respiration can be totally abolished by inducing the loss of mitochondrial DNA. We selected wild-type and vma2Δ mutants that had lost mitochondrial DNA (rho0 cells) and then determined whether the rho0 versions of the two strains were more or less sensitive to oxidative stress. As shown in Fig. 3A, loss of mitochondrial DNA did not affect the growth of either strain at pH 5. More importantly, the rho0 cells showed no improvement in their sensitivity to oxidative stress, as would be expected if the respiratory chain were the major source of the accumulating ROS (37). In fact, the vma2Δ rho0 strain was slightly more sensitive to hydrogen peroxide. We also compared DHR staining between wild-type and vma2Δ cells in the presence and absence of mitochondrial DNA, and the results are shown in Fig. 3B. Although the percentage of cells showing DHR staining appeared to be somewhat lower in both the wild-type rho0 and vma2Δrho0 cells, the difference from the corresponding rho+ cells was not sta-
tically significant. In contrast, there was a significant (p < 0.05) difference between the wild-type and vma2Δ strain, both for the rho+ and the rho0 strains. These results argue against the respiratory chain as the major source of endogenous oxidative stress in the vma mutants.

It is still possible that mitochondria contribute to oxidative stress in cells grown in nonfermentable carbon sources, where respiration is required and ROS production from the respiratory chain may be unavoidable. Like many strains that are sensitive to oxidative stress (32), vma mutants are unable to grow at typical concentrations of nonfermentable carbon sources, although it is possible to isolate metabolically normal mitochondria from the mutants (38). Supek et al. (39) reported that vma mutants were able to grow at lower concentrations of non-

FIGURE 2. V-ATPase deletion mutants show evidence of chronic oxidative stress. A, wild-type and BY4741 vma2Δ were stained with dihydrorhodamine 123 as described under “Experimental Procedures.” After 4.5 h staining, fields of cells were visualized using a ×40 objective, under Nomarski optics (DIC; left field of each set) and rhodamine fluorescence optics (right field of each set). B, wild-type and BY4741 vma2Δ cells in logarithmic phase growth were converted to spheroplasts and lysed as described under “Experimental Procedures.” Lysate proteins were reacted with dinitrophenyl hydrazine (+) using the OxyBlot kit or subjected to a mock derivatization (−). After derivatization, equal amounts of protein from each sample were loaded in each lane of an SDS-polyacrylamide gel, separated, and transferred to nitrocellulose. DNP labeling of carbonylated proteins was detected by immunoblotting with a rabbit anti-DNP polyclonal antibody followed by incubation with horseradish peroxidase-conjugated second antibody and color development using an enhanced chemiluminescence kit (Amersham Biosciences).

FIGURE 3. The electron transport chain may not be the major source of ROS in vma mutants grown in glucose. A, wild-type and BY4741 vma2Δ cells were depleted of mitochondrial DNA as described under “Experimental Procedures” to form rho0 cells, and the growth of the rho+ and rho0 cells on SC and SC containing the indicated concentration of H2O2 is compared. For each strain, 10-fold serial dilutions from liquid cultures of cells at identical density were spotted on the indicated plates. Cells were grown for 2 days at 30 °C. B, the percentage of both rho+ and rho0 cells stained with DHR was measured in two independent experiments, and the mean ± range of the two experiments is shown. C, the growth of wild type and vma2Δ mutants on YEPD, pH 5 (optimal growth conditions), YEP containing typical concentrations of nonfermentable carbon sources (3% glycerol plus 2% ethanol or 3% lactate), and YEP with lowered concentrations of nonfermentable carbon sources (3% glycerol only or 0.3% lactate) is compared as described in A.
fermentable carbon sources, also suggesting that defective respiration is not the cause of the petite phenotype in the vma mutants. We assessed the growth of the vma2Δ mutant at varied concentrations of two different nonfermentable carbon sources, glycerol/ethanol and lactate. As shown in Fig. 3C, the vma2Δ mutant cannot grow on 3% glycerol plus 2% ethanol or on 3% lactate, although the wild-type strain grows vigorously under both conditions. However, the vma2Δ mutant was able to grow in the presence of 3% glycerol without added ethanol or 0.3% lactate.

There are numerous defense systems against oxidative stress in yeast, including the cytosolic Cu/Zn-superoxide dismutase Sod1p, thioredoxin Trx2p and thioredoxin-dependent peroxidase Tsa1p, reduced glutathione (synthesized and regenerated by glutathione synthetase Gsh1p and glutathione reductase Grl1p, respectively), and the oxidative arm of the pentose phosphate pathway (33). Complementing these cytosolic defense systems are some similar proteins localized to the mitochondria, including a mitochondrial thioredoxin, Trx3p, and the manganese-dependent superoxide dismutase Sod2p. Mutations in each of these defense systems result in increased sensitivity to challenge with extracellular oxidant (1, 33). We reasoned that double mutants lacking both the V-ATPase and one of these defense systems result in increased sensitivity to challenge with oxidative stress in the vma mutants. We tested this hypothesis by crossing the vma2Δ mutant to haploid deletions in several major cytosolic and mitochondrial antioxidant defense systems, selecting diploids, and then sporulating the diploids and dissecting tetrads to obtain double mutant spores. Spores were allowed to germinate on YEPD medium buffered to pH 5, conditions that are optimal for growth of vma deletion mutants and that appear to minimize sensitivity to exogenous oxidative stress. Viable double mutant spores were obtained for crosses of vma2Δ trx2Δ, vma2Δ gsh1Δ, gsh1Δ sod2Δ, and zwf1Δ, and in Fig. 4A, the growth of several of the double mutant spores on SC and SC medium containing 0.5 mM H2O2 concentration is compared. In general, the double mutant spores are clearly more sensitive to hydrogen peroxide than the single mutants. Although double mutant spores were obtained by dissection on YEPD pH 5, the gsh1Δvma2Δ double mutant was not only unable to grow in the presence of low peroxide concentrations (data not shown) but also was unable to grow at all on supplemented minimal medium. This indicates a synthetic effect of the gsh1Δ and vma2Δ mutations that is manifest even in the absence of additional oxidative stress. All of the other mutations tested showed increased sensitivity to oxidative stress when combined with the vma2Δ mutation, but the increase in sensitivity appears to be more pronounced for the cytosolic defense system mutants shown in Fig. 4A, trx2Δ and gnr1Δ, than for the mitochondrial defense system mutants, trxr3Δ and sod2Δ.

In similar dissections of a diploid heterozygous for sod1Δ and vma2Δ, we were not able to obtain double mutant spores, as shown in Fig. 4B. This suggests that the two mutations are synthetically lethal, even on the YEPD, pH 5, plates used for dissection. However, both sod1Δ and vma2Δ have defects in spore germination, and this could prevent growth of otherwise viable spores. In order to bypass the germination defect, we transformed the vma2Δ mutant with a plasmid-borne copy of wild-
type VMA2, and then crossed, selected for diploids, and dissected tetrads. As shown in Fig. 4B, under these conditions, four-spore tetrads could be obtained. The VMA2-containing plasmid contained a URA3 marker, so we tested for the presence of the plasmid in each spore by determining growth on medium lacking uracil and also transferred the spores to medium containing 5-fluoroorotic acid, a negative selection for the URA3 marker. Two viable spores (boxed in Fig. 4B) contained both the vma2Δ and sod1Δ mutations and also showed some growth on 5-fluoroorotic acid, indicating that they were able to lose the VMA2 plasmid. These spores showed very slow growth and poor viability and could not be tested further for oxidant sensitivity. Therefore, sod1Δ vma2Δ double mutant spores can be obtained but have a very severe synthetic growth defect, even in the absence of an imposed oxidative stress. In contrast, sod2Δvma2Δ spores, shown for comparison in Fig. 4B, were readily obtained from tetrad dissection and grew well on YEPD, pH 5, suggesting that there is a much less severe synthetic defect between the mitochondrial superoxide dismutase mutant and the vma2Δ mutant.

Taken together, these results indicate that most of the major cytosolic mechanisms for handling oxidative stress are operating in the vma2Δ mutant. In fact, they are critical for its optimal growth, because double mutants exhibit increased sensitivity to low concentrations of H2O2 and poorer growth even in the absence of added oxidant in some cases. Therefore, it is unlikely that the sensitivity of the V-ATPase mutants to oxidative stress is coming from their inability to support one of these mechanisms, but instead, loss of V-ATPase activity must help to create an additional source of increased oxidative stress. Loss of mitochondrial protection systems aggravates the sensitivity of the vma mutants to oxidative stress but less severely than loss of the parallel cytoplasmic systems.

Microarray Analysis Indicates Dramatically Perturbed Metal Ion Homeostasis and Up-regulation of TSA2 in a vma2Δ Mutant—The overall transcriptional profile of a cell can provide critical insights into its physiological state. In an effort to better understand potential sources of oxidative stress in the vma mutants, we grew both wild-type cells and a congenic vma2Δ mutant to log phase in YEPD, pH 5, prepared RNA, and then performed microarray analysis to compare the transcriptional profiles of the two strains. Genes that were up-regulated or down-regulated 2.1-fold or more (calculated to be at the 99th percentile confidence level as described under “Experimental Procedures”) in the vma2Δ strain are listed in supplemental Table 1. These data were analyzed using the Saccharomyces Genome Database Gene Ontology (GO) Term Finder program to extract GO terms that are enriched in the up-regulated or down-regulated gene sets relative to the entire set of yeast genes on the microarray. The results from the set of up-regulated genes were particularly notable in the context of the oxidative stress sensitivity of the vma2 mutant. Table 1 lists the GO process terms that were calculated to have a p value (probability of random association) of <0.01 for this set of genes as well as the enrichment of each term in the data set relative to its representation in the genome. It is clear from this analysis that there is a highly significant enrichment for genes in two general categories: 1) metal ion (particularly iron) transport and homeostasis and 2) arginine/glutamine/ornithine biosynthesis and metabolism.

To begin to assess the extent to which the vma2Δ mutant is responding transcriptionally to oxidative stress, we compared the set of genes up-regulated or down-regulated by at least 4-fold in the vma2Δ strain to the published sets of genes changed by at least 4-fold in response to a grx5Δ mutation or 20-min treatment with 0.3 mM H2O2 (5, 40). The grx5 null mutant has been used as a model of yeast cells under chronic, modest oxidative stress (40). Grx5p is a mitochondrial glutaredoxin, and in its absence, cells accumulate carboxylated proteins even in the absence of applied oxidative stress (41), symptomatic of chronic oxidative stress (see Fig. 2). Transcriptional response to an acute challenge to H2O2 was measured by Gasch et al. (5) in the context of determining common features in response to environmental stress. Only 5323 genes were available in all three data sets for comparison, but of these, 264 genes showed a 4-fold change in at least one of the three data sets, and three-way correlations of these genes are shown in Fig. 5. There was very little correlation between the acutely stressed H2O2-treated cells and the chronically stressed grx5 mutant (R2 = 0.0038). The vma2Δ mutant data correlated better with each of these data sets, showing an R2 = 0.060 with the grx5 data and an R2 = 0.092 with the H2O2 treatment data. Taken together, these data indicate that the transcriptional response to loss of Vma2p has elements in common with responses seen in these models for chronic and acute oxidative stress but also has clear differences. Tabulated data used to generate Fig. 5 are shown in supplemental Table 2. As part of this analysis, we were also able to identify which genes were changed by at least 4-fold in more than one data set. Only eight genes were changed by at least 4-fold in more than one data set, and all were increased. Five of these, FIT3, FIT2, ARN1, ARN2, and YBR047w, have previously been implicated in iron and/or copper homeostasis (42, 43). Each of these genes was up-regulated by at least 2.9-fold in all three data sets, suggesting that they are strong common components.

Because of the potential for links between perturbed metal ion homeostasis and oxidative stress, we probed Northern blots of two independent RNA preparations from the wild-type and vma2Δ genes for several of the genes that are implicated in iron/copper homeostasis and that showed altered expression in the microarray. The results are shown in Fig. 6. The Northern blots show a clear up-regulation of mRNAs for FIT2, which encodes one of a family of cell wall mannoproteins that assist in iron uptake; SIT1 and ENB1, which both encode iron-siderophore transporters; FRE2, an iron reductase at the plasma membrane; HMX1, an ER-localized heme degradation enzyme required for recovery of iron from heme; and FET3, required for high affinity iron transporter at the plasma membrane (44–46). In contrast, the major high affinity transporter of copper at the plasma membrane, encoded by CTR1 (47), is down-regulated in the vma2Δ cells. Although most enzymes previously identified as up-regulated in response to an applied oxidative stress are not significantly up-regulated, there is a large up-regulation of TSA2, which encodes an isofrom of thioredoxin peroxidase.
Yeast V-ATPase Protects against Oxidative Stress

TABLE 1
GO process terms enriched in the set of up-regulated genes in vma2Δ mutant

Genes that were induced at least 2.1-fold (corresponding to the 99% confidence limit for the microarray data; see “Experimental Procedures”) were submitted to the GO Term Finder program for GO process terms at the Saccharomyces Genome Database. GO process terms with a p value of <0.01 are listed. The -fold enrichment was calculated by comparing representation of the term in the set of induced genes with its representation in the yeast genome. GO terms corresponding to identical sets of genes with identical p values are listed together and separated by a slash. GO terms corresponding to identical sets of genes that have different p values are listed with their respective p values and enrichment.

| GO process term                  | p value | -fold | Enrichment | Gene                                                                 |
|----------------------------------|---------|-------|------------|----------------------------------------------------------------------|
| Transport                         | 0.00868 | 1.6   |            | FIT2 SIT1FIT3 ARN2 FKE4 ENB1 ARN1 FKE2 VMB1 HXT5                    |
|                                  |         |       |            | CAC2 AGP2 CYC7 COS10 COT1 YHR048W ATG22 FKE3                       |
|                                  |         |       |            | MCH4 FET3 FTR1 PHO84 BAP2 AQY2 DIPS BCH2                            |
| Ion transport                     | 3.88E-07| 6.5   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE2 VMB1 HXT5                            |
| Cation transport                  | 5.85E-08| 7.8   |            | FET3 FTR1                                                           |
| Di- and trivalent inorganic cation transport | 8.73E-11 | 2.3   |            | FIT2 SIT1FIT3 ARN2 FKE4 ENB1 ARN1 FKE3                            |
| Metal ion transport               | 3.05E-10| 12.6  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE2 VMB1 HXT5                            |
| Transition metal ion transport    | 9.64E-12| 17.1  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Iron ion transport                | 5.18E-11| 28.3  |            | FET3 FTR1                                                           |
| High affinity iron ion transport  | 0.00328 | 23.9  |            | FIT2 SIT1FIT3 ARN2 FKE4 ENB1 ARN1 FKE3                            |
| Siderophore transport             | 4.57E-12| 53.1  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Siderophore-ion transport         | 1.15E-09| 59.8  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Homeostasis                       | 1.04E-06| 5.9   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Cell homeostasis                  | 1.78E-06| 6.3   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Cation homeostasis                | 5.00E-07| 7.1   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Metal ion homeostasis             | 1.16E-08| 10.4  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Di- and trivalent inorganic cation homeostasis | 3.21E-08| 11.1  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Transition metal ion homeostasis  | 1.07E-08| 12.5  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Iron homeostasis                  | 5.21E-08| 15.9  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Organic acid metabolism/carboxylic acid metabolism | 0.00018 | 2.9   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Nitrogen compound metabolism      | 5.54E-05| 3.5   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Amino acid metabolism             | 6.62E-05| 3.9   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Amino metabolism                  | 0.0001  | 3.5   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Amino acid and derivative metabolism | 0.00014 | 3.6   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Glutamine family amino acid metabolism | 5.77E-08 | 12.5  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Arginine metabolism/urea cycle intermediate metabolism | 2.50E-10 | 31.9  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Nonprotein amino acid metabolism  | 5.26E-07| 33.2  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Ornithine metabolism              | 3.72E-06| 39.8  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Amino acid biosynthesis           | 1.03E-05| 5.8   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Nitrogen compound biosynthesis/amino biosynthesis | 2.10E-05 | 5.3   |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Glutamine family amino acid biosynthesis | 2.33E-08 | 17.7  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Ornithine biosynthesis            | 0.00212 | 29.9  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Nonprotein amino acid biosynthesis | 0.00468 | 19.9  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Arginine biosynthesis             | 5.32E-10 | 41.8  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |
| Response to water/response to water deprivation/response to desiccation | 0.00814 | 14.9  |            | SIT1 ARN2 FKE4 ENB1 ARN1 FKE3 FTR1                                |

Overall, the Northern results parallel those from microarray analysis and help to validate that analysis (Fig. 6 and supplemental Table 1), although the microarray tended to underestimate the magnitude of the change in most cases. Comparative studies of quantitative reverse transcription-PCR and microarray methodologies have noted that although robust multiarray average-based normalization greatly reduces the false positive rate, it also tends to underestimate the magnitude of real changes compared with quantitative real time reverse transcription-PCR, particularly for low abundance transcripts (48, 49).

Many of the genes involved in iron and copper homeostasis are under control of the Aft1p and/or Aft2p transcription factors (50, 51). The set of genes under control of these transcription factors is collectively known as the Aft1p/Aft2p regulon. Up-regulation of the Aft1p/Aft2p regulon in the vma2Δ mutant was not entirely unexpected, because vma mutants have previously been shown to be iron-deprived, possibly as a result of failure to mature Fet3p, the multicopper oxidase required for high affinity iron transport, in an acidic compartment (52). If failure to mature Fet3p and the resultant iron deprivation and up-regulation of the Aft1p/Aft2p regulon is the source of the oxidative stress sensitivity in the vma mutants, then a fet3Δ mutant should be as sensitive as the vma2Δ mutant. Fig. 7 shows a direct comparison of the peroxide sensitivity of the fet3Δ mutant and vma2Δ. It is clear that the vma2Δ mutant is much more sensitive to peroxide stress than the fet3Δ mutant. We quantitated relative halo size for the fet3Δ mutant in two independent experiments (as shown for the vma2Δ mutant in Fig. 1D) and found that the mean halo size was 1.05- and 1.1-fold that of the wild-type strain in the presence of 2 and 5% H2O2, respectively. Based on these data, loss of Fet3p function cannot account for the sensitivity of the vma2Δ mutant to oxidative stress.

DISCUSSION

Sensitivity to Oxidative Stress Is a General Feature of vma Mutants—Genomic screens for deletion mutants sensitive to oxidative stress have frequently identified vma deletion mutants, but in some cases, these screens have identified only a
Yeast V-ATPase Protects against Oxidative Stress

We deliberately chose to assess sensitivity to oxidative stress in strains containing deletions in a V₁ sector subunit (vma2Δ) and a V₀ sector subunit (vma3Δ) that are both found in all yeast V-ATPases as well as the two V₀ subunit isoforms (vph1Δ and stv1Δ), each found in a subset of V-ATPases. We found that all were sensitive, suggesting that sensitivity is a general feature of loss of V-ATPase activity. The vma2Δ and vma3Δ mutants were more sensitive than the vph1Δ and stv1Δ mutants, but this is expected, because the latter two mutants only lack a subset of V-ATPase complexes. The vma3Δ mutant may be slightly more sensitive to peroxide (Fig. 1A) and other oxidants (data not shown) than the vma2Δ mutant. This difference is harder to explain, because both mutations abolish all V-ATPase activity. However, Vma3p has also been suggested to play a role in vacuole-vacuole fusion independent from V₁ subunits (53), and it is possible that an additional function could account for the small increase in sensitivity to oxidative stress. Taken together with the previous data, these results suggest that loss of V-ATPase function is responsible for the sensitivity to oxidative stress and that loss of function in both the vacuolar and Golgi/endosome compartments may contribute to this sensitivity.

Increased susceptibility to oxidative stress may also account for other characteristics of vma mutants. The inability of vma mutants to grow on nonfermentable carbon sources has long been rather puzzling, particularly since Ohya et al. (38) demonstrated that the mutant cells have normal rates of respiration as well as normal levels of several respiratory chain enzymes in isolated mitochondria. These experiments argued against the inability to synthesize respiratory enzymes (e.g. as a result of defects in metal ion trafficking or distribution (54)) as the source of the growth defect in the vma strains. However, many mutants sensitive to oxidative stress fail to grow on nonfermentable carbon sources, presumably because they cannot tolerate the levels of superoxide and other ROS produced as side products of respiration (7). We have also found that vma mutants are very slow to return to log phase growth after they have grown to high density.4 This is also characteristic of certain mutants sensitive to oxidative stress, because they poorly tolerate the diauxic switch that accompanies consumption of available glucose and the metabolic transition from fermentation to consumption of ethanol (55–58). The ability of the vma mutants to grow on low, but not high, concentrations of nonfermentable carbon sources (Fig. 4A) (39) may also be consistent with their sensitivity to oxidative stress. Supek et al. (39), who first observed this phenomenon, hypothesized that vacuolar function was required for storage (or possibly metabolism) of excess respiratory metabolites. These “excess metabolites” could be ROS produced as a side product of rapid respiration. However, the growth of the vma mutants on low concentrations of nonfermentable carbon sources also seems to be somewhat paradoxical, because there is general agreement that at high levels of respiratory activity lower levels of ROS are produced (36, 59). Further work will be necessary to fully understand the connections between mitochondrial function and oxidative stress in the vma mutants.

subset of the vma mutants (7), raising the possibility that resistance to oxidative stress is a “moonlighting” function of certain V-ATPase subunits rather than a general role of the V-ATPase.

**FIGURE 5.** Correlation of expression changes in the vma2Δ mutant, grx5Δ, and wild-type (wt) cells treated for 20 min with 0.3 mM H₂O₂.

Genes showing at least a 4-fold change of expression between vma2Δ and wild-type cells (this study), grx5Δ (a model of wild-type chronic oxidative stress), and wild-type cells (40) or wild-type cells with and without a 20-min treatment with 0.3 mM H₂O₂ (a model of acute oxidative stress (5)) were identified, and the expression of these genes among the three experiments was compared. 264 genes changed by at least 4-fold in at least one of the data sets and thus are included in the comparison. Log₂ changes for each of these genes are plotted against the other two and used to calculate the indicated R² value.

4 E. Milgrom, H. Diab, F. Middleton, and P. M. Kane, unpublished data.
Potential Sources of Oxidative Stress in vma Mutants—Although sensitivity to oxidative stress may account for the inability of the vma mutants to grow on standard concentrations of nonfermentable carbon sources, superoxide production from the respiratory chain, generally considered a major source of cellular ROS (60), does not account for the peroxide sensitivity of the vma2Δ/H9004 mutant. A number of different mutants that are sensitive to oxidative stress show improved growth in the absence of mitochondrial DNA (37, 61). Eviction of mitochondrial DNA from vma2Δ/H9004 mutant to form the vma2Δ/rho0 mutant did not improve the general growth properties of the mutant or its resistance to peroxide challenge (Fig. 4B). rho0 strains have previously been shown to have lowered resistance to peroxide in some cases (62), but the basis of this phenomenon is not well understood. There was relatively little decrease in ROS staining in the vma2Δ/rho0 strain, again suggesting that respiration is not the major source of ROS.

We reasoned that the vma mutants might be affecting one of the other major mechanisms for resistance to oxidative stress (e.g. by perturbing metal ion access for assembly of superoxide dismutase). Although we cannot completely eliminate this possibility, the strong synthetic phenotypes of mutants lacking both vma2Δ and several other mechanisms argue that loss of V-ATPase function creates an additional source of stress.

The strong up-regulation of the Aft1/Aft2 regulon in the vma2Δ/H11002 mutant also highlights the potential importance of the V-ATPase in metal ion homeostasis and suggests some possible sources of oxidative stress sensitivity. Davis-Kaplan et al. (52) previously showed that vma mutants have low levels of cellular iron that may be attributed to the requirement for an acidic post-Golgi compartment for insertion of the copper cluster into apo-Fet3p. Iron deprivation and/or loss of Fet3p function has also been associated with increased sensitivity to other transition metals because of up-regulation of less specific transporters (63). Because some of these metals are redox-active, they could contribute to oxidative stress. However, we do not believe that the iron deprivation that occurs in vma mutants is solely responsible for the oxidative stress. First, if loss of Fet3p maturation were the root cause of the sensitivity of vma2Δ to oxidative stress, then the fet3Δ mutant should be as sensitive to oxidative stress as the vma2Δ mutant, but it is not (Fig. 7). Second, we found that although the addition of iron and copper to a number of mutants exhibiting a Vma− phenotype improved their growth, it did not improve the growth of the vma mutants (16). In contrast, the addition of excess iron reduced the toxicity

H2O2: 2% 5%
wt fet3Δ vma2Δ

FIGURE 7. Loss of Fet3p function does not result in strong sensitivity to oxidative stress. Halo assays for wild-type cells and the congenic fet3Δ and vma2Δ mutants were prepared as described in the legend to Fig. 1 and under “Experimental Procedures,” with 2 and 5% H2O2, applied to the filter disk. Lawns of cells were grown for 2 days.
of other metals by competition under conditions where less specific transporters were up-regulated (63). In addition, although the Aft1/Aft2 regulon clearly has an iron-sensitive input, it is also induced in conditions other than direct iron deprivation, including chronic oxidative stress (40). In fact, cells become somewhat more sensitive to oxidative stress in the absence of Aft1p and much more sensitive in the absence of both Aft1p and Aft2p (50). This suggests that up-regulation of the Aft1/Aft2 regulon could be a response to the oxidative stress in the vma2Δ mutant rather than a cause.

Significantly, in the vma2Δ mutant, we did not see a dramatic up-regulation of the oxidative stress response genes controlled by Yap1p and Skn7p, which are strongly up-regulated in response to an acute oxidative stress (5, 64). Consistent with this, we did not see a strong nuclear concentration of GFP-Yap1p (25) in the vma2Δ mutant grown in the absence of applied oxidant, although GFP-Yap1p was translocated to the nucleus with H2O2 addition (data now shown). Microarray analysis of the grx5Δ mutant indicated little up-regulation of Yap1- or Skn7-dependent genes but did show a strong up-regulation of the Aft1/Aft2 regulon, similar to that seen in the vma2Δ mutant (40). However, the comparison with microarray data from cells treated for 20 min with H2O2 (Fig. 5) does suggest that there are at least some common features between transcriptional response to vma2Δ and an acute oxidative challenge with H2O2.

In conclusion, we do not fully understand the source of the chronic oxidative stress seen in the vma2Δ mutant, but we can propose several potential sources. First, it is entirely possible that improper distribution or utilization of one or more metals contributes to the oxidative stress, although a simple explanation based on iron deprivation does not seem sufficient. In a recent genomic dissection of the S. cerevisiae "ionome," the V-ATPase was identified as a major player in uptake and distribution of multiple different metals (15). It is also possible that altered pH homeostasis in the vma mutants leads to an environment that extends the lifetime of certain ROS or otherwise changes their metabolism. Regardless of the mechanism, the V-ATPase appears to play a unique and essential role in resistance to oxidative stress that may be important in other eukaryotic cells as well.

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