Alternative Mechanisms of Vacuolar Acidification in H\(^+\)-ATPase-deficient Yeast*

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Acidification of the endosomal/lysosomal pathway by the vacuolar-type proton translocating ATPase (V-ATPase) is necessary for a variety of essential eukaryotic cellular functions. Nevertheless, yeasts lacking V-ATPase activity (Δvma) are viable when grown at low pH, suggesting alternative methods of organellar acidification. This was confirmed by directly measuring the vacuolar pH by ratio fluorescence imaging. When Δvma yeasts were cultured and tested in the acidic conditions required for growth of V-ATPase-deficient mutants, the vacuolar pH was 5.8. Fluid-phase pinocytosis of acidic extracellular medium cannot account for these observations, because the V-ATPase-independent vacuolar acidification was unaffected in mutants deficient in endocytosis. Similarly, internalization of the plasmalemmal H\(^+\)-ATPase (Pma1p) was ruled out, because overexpression of Pma1p failed to complement the Δvma phenotype and did not potentiate the vacuolar acidification. To test whether weak electrolytes present in the culture medium could ferry acid equivalents to the vacuole, wild-type and the Δvma yeasts were subjected to sudden changes in extracellular pH. In both cell types, the vacuoles rapidly alkalinized when external pH was raised from 5.5 (the approximate pH of the culture medium) to 7.5 and re-acidified when the yeasts were returned to a medium of pH 5.5. Importantly, these rapid pH changes were only observed when NH\(_4\)\(^+\), routinely added as a nitrogen source, was present. The NH\(_4\)\(^+\)-dependent acidification was not due to efflux of NH\(_3\) from the vacuole, as cells equilibrated to pH 7.5 in the absence of weak electrolytes rapidly acidified when challenged with an acidic medium containing NH\(_4\)\(^+\). These findings suggest that although NH\(_3\) can act as a cell-permeant proton scavenger, NH\(_4\)\(^+\) may function as a protonophore, facilitating equilibration of the pH across the plasma and vacuolar membranes of yeast. The high concentration of NH\(_4\)\(^+\) frequently added as a nitrogen source to yeast culture media together with effective NH\(_4\)\(^+\) transporters thereby facilitate vacuolar acidification when cells are suspended in acidic solutions.

¹ The abbreviations used are: V-ATPase, vacuolar-type H\(^+\)-ATPase; PMA, plasma membrane H\(^+\)-ATPase; pH\(_{vac}\), vacuolar pH; BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; AM, acetoxymethyl ester; MES, 3-(N-morpholino)ethanesulfonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DIC, differential interference contrast; PAGE, polyacrylamide gel electrophoresis.
Δνma mutants could result from internalization of the plasma membrane H+-ATPase (Pma1p), a moneric protein that normally extrudes protons from the cytosol to the surrounding environment (21, 22). If internalized in its active state, Pma1p might contribute to the acidification of intracellular organelles. Finally, when yeasts are grown in rich media at low pH, acidification of the vacuole may result simply from the passive leakage of extracellular weak acids, which could reach and dissociate in the endosomal or vacuolar lumen.

In this report, we developed a sensitive microspectroscopic method to determine the pH within vacuoles of intact Saccharomyces cerevisiae. By using this approach, we proceeded to compare the pH of vacuoles from wild-type and Δνma mutant yeast, and we analyzed the mechanisms underlying the partial acidification observed in V-ATPase-deficient mutants.

**EXPERIMENTAL PROCEDURES**

**Materials and Media**

2,7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM) was obtained from Molecular Probes Inc. (Eugene, OR). Bafilomycin-A1, concanamycin A, and concanavalin A were from Sigma. YPD medium contained 1% yeast extract, 2% Bacto-peptone (BCECF-AM) was obtained from Molecular Probes Inc. (Eugene, OR). Yeast lysates from SF838-5A (ΔvmaΔ) transformed with YCP2HSE-PMA1 (kindly supplied by Dr. T. Stevens, University of Oregon). The following yeast strains were used: BJ926, MATα/a trp1/1+his3/pdr1/1+his3/Δα::ADH1::URA3 (kindly supplied by Dr. T. Stevens, University of Oregon). The plasmid Yc2HSE-PMA1 (kindly provided to us by Dr. C. Slayman, Yale University) contains the full-length PMA1 gene preceded by two tandem copies of the heat shock element (23).

**Strains and Plasmids**

The following yeast strains were used: BJ926, MATa/a trp1/1+ +/his3/pdr1/1+pdr3/3/pdr4/3/Δα::PDR31::NIPH1/2/Δα::NIPH3/2/Δα::NIPR2/1::URA3 (kindly supplied by Dr. T. Stevens, University of Oregon). The plasmid Yc2HSE-PMA1 (kindly provided to us by Dr. C. Slayman, Yale University) contains the full-length PMA1 gene preceded by two tandem copies of the heat shock element (23).

**Growth and Labeling of Yeast**

Strains were grown in rich medium (YPD) or minimal medium for transformed yeast as described before in Ausubel et al. (24). Buffered medium was prepared by the addition of 50 mM MES to either rich or minimal media, and the pH was adjusted to the indicated value with NaOH. YPD plates supplemented with 100 mM CaCl2, 4 mM ZnCl2, or buffered to pH 7.5 with 50 mM MES-Tris were prepared as described in Manolson et al. (25). Growth phenotypes of Δvma and Δvma-YPc2HSE-PMA1 were assessed as described before (25), after incubating the plates at 30 or 37 °C for 3 days for Δvma (5.5), 100 mM CaCl2, and pH 7.5-buffered plates, and for 5 days for plates containing 4 mM ZnCl2.

For vacuolar pH determinations, yeasts were harvested at 107 cells/ml and resuspended in rich medium containing 50 μM BCECF-AM and incubated at the specified temperature for 15–30 min. For measurements of cytosolic pH, the cells were loaded in rich medium buffered to pH 7.5. Yeasts were then sedimented, washed three times in rich medium, resuspended at 2 x 107 cells/ml in the indicated synthetic growth media, and used immediately for imaging.

**Video Microscopy and pH Imaging**

**Preparation of Cells**—For imaging, 100 μl of the BCECF-loaded yeast suspension was plated onto glass coverslips that had been pre-coated with concanavalin A (Sigma) as described (26). The coverslips were inserted into a temperature-controlled perfusion chamber (Medical Systems Corp., Greenvale, NY) and placed on the stage of an inverted microscope (Zeiss, Germany). Imaging acquisition proceeded immediately after the addition of 1 ml of the appropriate recording solution to the chamber. Yeast pH was recorded in the synthetic growth media described above, as normal growth media, or YPD produced background autofluorescence.

**Fluorescence Microscopy**—Ratio fluorescence imaging was performed as described (27), using a 60 x/1.25 Neofluor objective (Zeiss), a 75-watt Xenon epifluorescence lamp, and a shutter/filter-wheel assembly for wavelength selection (Sutter Instruments, Novato, CA). Images were acquired on a 1317 x 1035 pixels cooled digital CCD camera running at 1 MHz (Princeton Instruments, Trenton, NJ). Image acquisition and excitation filter selection was controlled by the Metafluor software (Universal Imaging, West Chester, PA). Red illumination allowed concurrent visualization of the cells by differential interference contrast (DIC), using a separate video camera (Dage-MTI, Michigan City, IN).

The bright fluorescence of BCECF at both excitation wavelengths (440 and 490 nm) and the use of high transmission objectives allowed to image the fluorescence of yeast vacuoles at the full resolution of the CCD array (i.e., no binning, pixel size 0.1 x 0.1 μm; e.g., Fig. 1). For the acidification experiments, images were acquired every 4 s (4 x 4 binning; pixel size = 0.4 x 0.4 μm) in order to decrease exposure time and minimize photobleaching and possible phototoxicity. The 490 and 440 nm fluorescence images were corrected for shading to compensate for uneven illumination, and the background was subtracted and a threshold of 3 times the value of the background noise (root mean squared) was applied before obtaining a pixel-by-pixel ratio of the two images. Sub-threshold pixels were neither displayed nor used for subsequent analysis to prevent artifacts caused by ratiointo zero-values.

**Calibration**—At the end of each experiment, a calibration curve of fluorescence ratio versus pH was obtained in situ by sequentially perfusing the cells with media containing 50 mM MES, 50 mM Hepes, 50 mM KCl, 50 mM NaCl, 0.2 mM ammonium acetate, 10 mM NaN3, 10 mM NH4OH, and 2-deoxyglucose, 50 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP), buffered at 4 different pH values ranging from 5.0 to 7.0 with NaOH as described previously (17).

**Image Processing**—An automated procedure was implemented to produce pH histograms or time graphs from the image data, using the pH-independent (440 nm) image to define individual cells. A small percentage of the yeast (<5%) had sustained cellular damage and displayed a bright fluorescence (>100-fold the normal intensity). These cells were excluded from the analysis by applying a high intensity threshold, whereas a low intensity threshold was used to define the edges of the vacuole. The image was then binarized, and the fluorescent objects were outlined by the computer (skeltonization). A size criterion was applied to retain only objects within ± 2 S.D. of the average size of yeast vacuoles. The average fluorescence ratio of individual yeast vacuoles was then calculated and converted to pH. This procedure allowed us to analyze an average of 20 yeasts per image, thus producing statistically significant information from images acquired within short (<10 min) intervals.

**Immunoblotting**

Yeast lysates from SF838-5A (ΔvmaΔ) transformed with YCP2HSE-PMA1 were prepared by growing cells at 30 °C to mid-log phase and then shifting to 37 °C for 1 h. Cells were harvested, washed, and resuspended in 63 mM Tris-HCl, pH 6.8, 1% SDS, 0.6 mM β-mercaptoethanol, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A together with an equal volume of acid-washed glass beads. Samples were vortexed 3 times for 30 s at 4 °C. Extracts were heated at 55 °C for 10 min, and cellular debris was removed by centrifugation for 5 min at 15,000 x g, at 4 °C. Equal amounts (50 μg) of protein were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against Pma1p (kindly supplied by Dr. J. Teem, Florida State University). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**RESULTS**

**BCEF Accumulates in the Yeast Vacuole**—The fluorescein derivative BCECF has been used extensively to measure the cytosolic pH in a variety of cell systems. When added to animal cells in its esterified precursor form, BCECF accumulates in cytosolic pH in a variety of cell systems. When added to animal cells in its esterified precursor form, BCECF accumulates in cytosolic pH in a variety of cell systems. When added to animal cells in its esterified precursor form, BCECF accumulates in cytosolic pH in a variety of cell systems.

Regardless of the underlying mechanism, this observation provided a conventional method to determine the pH within vacuoles of intact Saccharomyces cerevisiae.
ient and reproducible method for the noninvasive measurement of vacuolar pH. By using a high intensity threshold to exclude unwanted signals, we could readily measure the vacuolar pH by ratio fluorescence imaging (Fig. 1, central panel). BCECF was found to localize to the vacuole not only in wild-type yeasts but also in V-ATPase-deficient mutants (Fig. 1). In both types of cells the fluorescent probe was retained in the vacuole for extended periods; loss of BCECF was insignificant for approximately 1 h at room temperature.

**Calibration of Fluorescence Ratio Versus pH**—The procedure of Preston et al. (17) was used to manipulate and calibrate the vacuolar pH. The method uses solutions containing a metabolic inhibitor (deoxyglucose) to preclude regulation by endogenous transporters, a protonophore (CCCP) to increase H+ permeability across membranes, and very high concentrations of permeant weak electrolytes (0.2 M ammonium acetate), expected to mobilize and equilibrate acid equivalents across membranes. To validate the effectiveness of this pH-clamping procedure, the behavior of BCECF within the vacuole was compared with that observed in vitro, solubilizing the free acid of BCECF in calibration solutions of varying pH in the absence of yeast. Typical results comparing the pH dependence of the fluorescence ratio are illustrated in Fig. 2. As shown in Fig. 2A, although the dynamic range of the signal was reduced by 1.5-fold in situ, both titration curves had comparable sigmoidal shape and yielded similar values for the apparent pKa of BCECF, namely 6.50 in vitro and 6.65 in situ. A reduced dynamic range in situ is invariably observed in measurements with fluorescent dyes (30) and in this case probably reflects the interaction of BCECF with vacuolar constituents. We also found that the fluorescence ratio of the population distributed normally around the mean, that all the cells in the population responded to the calibration procedure (Fig. 2B), and that the pH calibration curves were identical for wild-type and Δvma4 yeasts (not shown). Based on these cumulative data, BCECF was deemed to be an adequate probe for quantitative measurements of vacuolar pH, and the in situ calibration procedure was used hereafter to convert the measurements of fluorescence ratio to luminal pH.

**V-ATPase-deficient Yeasts Have Acidic Vacuoles When Grown in Acidic Media**—Δvma4 yeasts cannot grow in alkaline-buffered media, pH 7.5, but grow optimally in media buffered to pH 5.5. If vacuolar acidification is essential for survival, this observation might suggest that yeasts are able to acidify their vacuoles in acidic media but not in media buffered at pH 7.5.
Both wild-type and Δvma yeast were grown overnight at pH 5.5, loaded with BCECF under the same conditions, and transferred to synthetic growth medium for measurement of fluorescence. Synthetic medium, as opposed to the conventional rich growth medium (yeast extract, peptone, dextrose), was used for pH determinations because the latter solution displayed a high degree of auto-fluorescence. Fig. 3A shows a typical distribution of vacuolar pH (pHvac) values of wild-type and Δvma yeast when recorded in medium of pH 5.5; although more alkaline than the wild-type yeast vacuoles (average pHvac = 5.45), the mutant yeast vacuoles are nevertheless considerably acidic (average pHvac = 5.9). When transferred to a medium of comparable composition buffered to pH 7.5, vacuoles of both wild-type and mutant cells became more alkaline (Fig. 3B). However, the wild-type cells underwent a comparatively minor pH shift, reaching steady state at a level (pHvac = 5.9) that is considerably more acidic than that of the medium. By contrast, the vacuolar alkalization was much more pronounced in V-ATPase-deficient cells (average pHvac = 7.05). This differential behavior is likely due to the activity of the V-ATPase, a notion that was validated using bafilomycin. Wild-type cells incubated at pH 5.5 in the presence of this V-ATPase antagonist equilibrated at a pHvac = 6.06, which was indistinguishable from that observed in the Δvma mutants. At pH 7.5, bafilomycin increased the steady state pH of wild-type cells by >1.3 pH units. Jointly, these observations suggest that Δvma mutants or cells in which the V-ATPase has been pharmacologically ablated are able to acidify their vacuoles only when grown at pH 5.5. In growth media buffered to pH 7.5 the vacuoles of Δvma yeast are no longer acidic, which may account for their inability to grow under such conditions.

The Plasma Membrane ATPase Does Not Contribute to Vacuolar Acidity—Yeast possess two distinct types of H+-pumping ATPases, the endomembrane V-type pump and the plasma membrane P-type pump. The latter is present in the plasma membrane, where it extrudes H+ from the cytosol, regulating intracellular pH and generating a transmembrane proton-motive force that is utilized by the yeast to translocate substrates by coupled transport (reviewed in Refs. 21, 22, and 31). In yeast, the plasma membrane H+-ATPase is encoded by two PMA (Plasma membrane H+-ATPase) genes, PMA1 and PMA2 (32, 33). Only the PMA1 gene is constitutively expressed and essential to growth (32). Because the lumen of the endocytic pathway is topologically equivalent to the extracellular space, endocytosis of the plasma membrane H+-ATPase, possibly during the normal degradative cycle of the pump, could in principle contribute to vacuolar acidification. Alternatively, Pma1p could be mistargeted to the vacuole during its synthesis in the Δvma mutant, due to alterations in the pH of the trans-Golgi. Accelerated internalization or synthesis of these pumps, or the increased availability of substrate, could account for the ability of the Δvma mutants to partially acidify their vacuole at acidic extracellular pH.

Gene disruption cannot be used to ascertain the role of Pma1p in vacuolar acidification, since the gene encoding this pump, PMA1, is essential. Instead, it was more practical to overexpress PMA1 to assess whether this would accentuate vacuolar acidification and possibly complement the Δvma phenotype, enabling the cells to grow at alkaline pH. To this end, PMA1 was placed under the control of two heat shock elements (YCp2HSE-PMA1) expected to increase the level of expression of Pma1p when transformed yeasts are grown at 37 °C (23). To ensure that growth at 37 °C did not affect the level of expression of Pma1p, lysates from transformed yeasts grown at 30 and 37 °C were prepared, and equal amounts of protein were analyzed by SDS-PAGE. Following transfer to nitrocellulose, immunoblots were probed with monoclonal antibodies to Pma1p. As shown in Fig. 4, yeasts grown at 37 °C were found to overexpress Pma1p.
Table I

Overexpression of Pma1p does not complement \( \Delta \text{uma} \) growth phenotypes

| Phenotype                  | pH \( \text{vac} \) | \( \Delta \text{uma} \) (30 °C) | \( \Delta \text{uma} \)-PMA1 (30 °C) | \( \Delta \text{uma} \) (37 °C) | \( \Delta \text{uma} \)-PMA1 (37 °C) |
|----------------------------|---------------------|-------------------------------|-----------------------------------|-------------------------------|-----------------------------------|
| Wild Type (WT)             | 5.5                 | +                             | +                                 | +                             | +                                 |
| \( \Delta \text{uma} \) (30 °C) | 5.5                 | -                             | -                                 | +                             | +                                 |
| \( \Delta \text{uma} \)-PMA1 (30 °C) | 5.5                 | -                             | -                                 | +                             | +                                 |
| \( \Delta \text{uma} \) (37 °C) | 5.5                 | -                             | -                                 | +                             | +                                 |
| \( \Delta \text{uma} \)-PMA1 (37 °C) | 5.5                 | -                             | -                                 | +                             | +                                 |

Complementation of \( \Delta \text{uma} \) phenotypes by Pma1p overexpression was tested by growth in media supplemented with \( \text{Ca}^{2+} \) or \( \text{Zn}^{2+} \) or buffered to alkaline pH (Table I). As reported earlier \( \Delta \text{uma} \) strains were unable to grow at high pH or in the presence of 100 mM \( \text{Ca}^{2+} \) or 4 mM \( \text{Zn}^{2+} \), presumably because removal of excess cations from the cytosol requires transport into the vacuole in exchange for luminal \( \text{H}^+ \). Acidification of the vacuolar lumen by the overexpressed Pma1p could result in significant growth under these conditions, despite the absence of functional V-ATPases. However, as documented in Table I, YCp2HSE-PMA1-transformed yeasts were unable to grow under these conditions at either 30 or 37 °C. This was not due to toxicity associated with overexpression of Pma1p, since the cells grew normally at pH 5.5. Instead, these observations suggest that Pma1p does not contribute to vacuolar acidification.

This was confirmed by direct fluorimetric measurement of pH\(_\text{vac}\) (Fig. 5). Overexpression of Pma1p in a \( \Delta \text{uma} \) mutant (\( \text{uma}+ \) PMA1, grown at 37 °C) did not affect the vacuolar pH under any of the conditions tested. When measured in media of pH 5.5, pH\(_\text{vac}\) was 5.9 in the untransformed \( \Delta \text{uma} \) cells, as well as in the transformants, whether they were grown at or below the inductive temperature. Similarly, when the cells were bathed in media of pH 7.5, the pH of the vacuoles averaged 7.0 in all cases (Fig. 5). Although we cannot exclude that \( \Delta \text{uma} \) mutants might also express PMA2 in addition to PMA1, the two isoforms are expected to be functionally equivalent. Because overexpression of Pma1p had no effect on the pH of the vacuole, nor was it able to complement even partially the \( \Delta \text{uma} \) phenotypes, we concluded that plasmalemmal pumps are unlikely to contribute significantly to vacuolar acidification.

Endocytosis Does Not Contribute to Vacular Acidification—Munn and Riezman (20) showed that mutations that disable the endocytic pathway are synthetically lethal with \( \Delta \text{uma} \) mutations. This finding supports the notion, originally proposed by Nelson and Nelson (15), that internalization of acidic extracellular medium by fluid-phase endocytosis may contribute to vacuolar acidification. To assess the contribution of endocytosis to vacuolar acidification in V-ATPase-deficient yeast, strains of yeasts bearing a temperature-sensitive mutation in endocytosis (\( \text{end}4 \)) were subjected to fluorescence imaging. When such yeasts are grown at 37 °C for 30 min, both fluid-phase and receptor-mediated endocytosis are blocked (34). Following growth at the permissive temperature, \( \text{end}4 \) was treated at 30 or 37 °C for approximately 30 min. During this period the yeasts were also labeled with BCECF and then immediately used for determination of pH\(_\text{vac}\). To evaluate the role of pinocytosis of acidic medium, without the confounding effects of the V-ATPase which is present and active in the \( \text{end}4 \) mutants, the cells were suspended in media containing bafilomycin. As shown in Fig. 6, cells suspended at pH 7.5 in the presence of the V-ATPase antagonist had pH\(_\text{vac}\) values that were similar to those of \( \Delta \text{uma} \) mutants, confirming the effectiveness of bafilomycin (compare the top and bottom panels). More importantly, the vacuolar acidification that occurs when \( \Delta \text{uma} \) cells are transferred to pH 5.5 medium was also observed in the \( \text{end}4 \) mutants incubated at the restrictive temperature (i.e., 37 °C). In fact, no difference in pH\(_\text{vac}\) was noted between \( \text{end}4 \) cells grown at 30 or 37 °C and tested in acidic media, regardless of the presence of bafilomycin (Fig. 6). In the absence of the inhibitor, however, the \( \text{end}4 \) cells became acidic even when maintained at pH 7.5 (Fig. 6), due to proton pumping by the V-ATPase. Parallel experiments demonstrated that the endocytosis defect characteristic of \( \text{end}4 \) mutants persisted at low pH. Fluid phase endocytosis, monitored as the uptake of Lucifer yellow, was markedly decreased in \( \text{end}4 \) cells grown at the restrictive temperature, regardless of the external pH (89% inhibition at pH 5.5 versus 91% inhibition at pH 5.5). Because nearly complete inhibition of endocytosis had little effect on pH\(_\text{vac}\) at pH 5.5, we concluded that delivery of acid equivalents to the vacuole does not occur by fluid-phase endocytosis of acidic medium.

Role of Weak Electrolytes on Vacular pH—During the course of the experiments described above, we noted that although wild-type yeasts maintained an acidic vacuolar pH in the steady state when grown in alkaline media, they underwent a transient alkalinization when transferred from the medium at pH 5.5 to medium at pH 7.5. This phenomenon is illustrated in Fig. 7, where the course of re-acidification is also shown. Restoration of the acidic pH is ostensibly due to the activity of the
NH₄⁺-mediated Acidification of the Yeast Vacuole

FIG. 6. Blocking endocytosis does not affect vacuolar acidification. Yeasts with a temperature-sensitive endocytosis defect (end4-1) and V-ATPase-deficient yeasts (SF838-5A; top panel) were equilibrated for 1 h in synthetic medium buffered to pH 7.5, and vacular pH was measured by ratio imaging (open bars). The recording solution was then changed to synthetic medium buffered to pH 5.5, and the yeasts were imaged again after ~5 min (shaded bars). When indicated (+baf), 5 μM bafilomycin-A₁ was added to the recording solution to block endogenous V-ATPase activity in the end4-1 mutant.

V-ATPase, since it is not observed in the Δoma yeast (Fig. 7) and is inhibited by bafilomycin (not illustrated).

We sought an explanation for the transient alkalization noted when changing the pH of the medium. The rapid nature of the changes suggested permeation of base equivalents into the vacuole. In this context, it is noteworthy that both rich and synthetic growth media contain 38 mM ammonium (as (NH₄)₂SO₄) as a nitrogen source. We considered the possibility that ammonia (NH₃), which is in equilibrium with ammonium (NH₄⁺), could permeate the plasma and vacuolar membranes and act as a base equivalent as it becomes protonated in the acidic vacuolar lumen (see the scheme in Fig. 9). Because at identical [NH₄⁺] the concentration of NH₃ is ~100-fold higher at pH 7.5 than at pH 5.5, increased entry of the base is expected during the transition from acidic to alkaline medium, with net consumption of vacular H⁺. To test this hypothesis, cells were grown in rich (NH₄)₂SO₄-containing medium at pH 5.5, loaded with BCECF, and then transferred acutely to a solution with identical pH and comparable osmolarity but devoid of NH₄⁺ (substituted by Na⁺). To circumvent any confounding effects due to the V-ATPase, Δoma cells were used. In the absence of NH₄⁺, replacing the pH 5.5 medium with medium at pH 7.5 failed to alkalinize the vacuole, which contrasts with the behavior noted in the presence of the weak base (Fig. 7B). Note that the starting pHvac was lower in the NH₄⁺-free medium than in the complete synthetic medium of comparable pH. This probably reflects efflux of the vacuolar ammonium accumulated during growth, the dissociation of NH₄⁺ leaving residual H⁺ within the vacuole during the transition to the NH₄⁺-free solution.

Because weak electrolytes can seemingly alkalinize the vacuole when the pH of the medium is increased, we considered the possibility that a similar mechanism may underlie the partial acidification of the vacuole in cells suspended at pH 5.5. As shown in Fig. 7B, cells that became alkaline upon transfer from medium pH 5.5 to 7.5 rapidly acidified to their initial pHvac when the acidic medium was restored. Importantly, this acidification was observed only when NH₄⁺ was present in the solution.

In the experiment of Fig. 7B, acidification of the vacuole during shift from pH 7.5 to 5.5 could be explained by an efflux of NH₃, caused by the sudden imposition of an outward NH₃ gradient as the weak base concentration decreases at lower pH. Therefore, it is unclear whether exit of the base or entry of the conjugated acid is responsible for the net H⁺ (equivalent) flux. To define unambiguously whether NH₄⁺ can ferry H⁺ into the vacuole, cells were initially equilibrated in alkaline medium devoid of NH₄⁺, in the presence of bafilomycin to preclude V-ATPase activity. The cells were then rapidly switched to media of pH 5.5, with or without NH₄⁺. As illustrated in Fig. 8, in the presence of the conjugated acid vacuolar pH dropped rapidly and equilibrated near pH 6.5. By contrast, pH changed very little in the absence of NH₄⁺, despite the large inward H⁺ gradient. The data of several hundred determinations are collected in Fig. 8B. When maintained at external pH 7.5 in the
absence of NH$_4^+$ (with bafilomycin), pH$_{vac}$ averaged 7.35, and only a small decrease was seen after 2 h of incubation at pH 5.5 in the absence of NH$_4^+$. In contrast, pH$_{vac}$ reached 6.53 when NH$_4^+$ was present. The rapid acidification shown in Fig. 8 cannot be accounted for by net NH$_3$ efflux, since the base was absent from the cell interior at the time of the pH change. Therefore, the most parsimonious explanation of these data is that ammonium (NH$_4^+$) acts as a weak conjugated acid, mediating the inward delivery of H$^+$ (equivalents) across the plasma and vacuolar membranes.

NH$_4^+$ Permeates the Plasma Membrane and Acidifies the Cytosol—In order for NH$_4^+$ to deliver acid equivalents to the vacuole, it must first permeate the yeast plasma membrane. Such permeation of the protonated species is expected to induce a cytosolic acidification. To assess this possibility, we implemented a strategy to measure the cytosolic pH of yeast. Earlier studies indicated that pH-sensitive fluorescent dyes accumulated in the cytosol when yeasts were loaded with the precursor esters under alkaline, rather than acidic conditions (18, 35). This pH-specific targeting probably reflects the different pH optima of cytosolic and vacuolar esterases and was more pronounced with the neutral dye carboxy-SNARF-1 than with the more acidic BCECF. Consistent with this observation, we observed that, when cells were loaded under alkaline (pH 7.5) media, SNARF-1 readily loaded the yeast cytosol. Similarly, BCECF, which accumulated into the vacuole under our standard loading conditions (Fig. 1), yielded intense cytosolic staining in ~30% of cells loaded in alkaline medium (Fig. 9A, top panels). The cytosolic staining persisted when cells were subsequently challenged with acidic extracellular media (Fig. 9B), confirming that, once cleaved by cytosolic esterases, the dye was not transported into the vacuole but remained localized in the cytosol. The bright cytosolic labeling produced by BCECF could be easily separated from the vacuolar signal by applying an intensity threshold, combined with a size criterion to reject contaminating vacuoles (Fig. 9A, bottom panels). This enabled us to measure the pH of the yeast cytosol by ratio imaging, employing the same dye and calibration procedure used for the vacuolar measurements. As shown in Fig. 9C, wild-type and Δuma yeasts had similar cytosolic pH when maintained in alkaline, pH 7.5, medium containing NH$_4^+$. More importantly, the cytosol rapidly acidified in both cell types when challenged with acidic media containing NH$_4^+$ (Fig. 9D). This effect was more rapid than the NH$_4^+$-mediated acidification of the vacuole (e.g. Fig. 8), suggesting that NH$_4^+$ sequentially permeates the plasma and then the vacuolar membrane of yeast.

**Discussion**

Video Imaging of BCECF Is an Accurate and Convenient Method for Measuring Vacuolar and Cytosolic pH—$^{31}$P NMR and dual excitation flow cytometry of 6-carboxyfluorescein diacetate had been used earlier to measure the pH of yeast vacuoles (13, 17, 19). Values of 6.1 (13) and 6.2 (17) were obtained using 6-carboxyfluorescein, and a pH in the range of 5.5 to 6.0 was estimated using $^{31}$P NMR (19). Although useful, these methods have limitations. Acquisition of NMR data is slow, and it is difficult to make acute changes in the composition of the medium. Moreover, compartments other than the vacuole contribute to the resonance signal measured. A similar problem plagues the flow cytometric method since, as shown here, although esters of fluorescein derivatives accumulate in the vacuole, they are not restricted to this compartment. Indeed, they were designed to accumulate in the cytosol (36). In the case of video imaging, definition of the regions of interest together with thresholding of unwanted signals ensures that the vacuolar or cytosolic pH is selectively measured. Moreover, by adhering the yeast to a coverslip mounted in a perfusion chamber, rapid solution changes are possible.

Vacuolar Acidification Is Indispensable in S. cerevisiae—In eukaryotic cells, V-ATPases are responsible for generating transmembrane electrochemical gradients within several organelles. These gradients are thought to be critical for essential cellular functions such as sorting and processing of proteins, receptor recycling, and the control of vesicular traffic. In *N. crassa*, the gene encoding the 70-kDa catalytic ATP-binding subunit, *uma-1*, was shown to be essential for survival, demonstrating that V-ATPase activity is indispensable in this ascomycete fungus (37). In contrast, the filamentous fungi *Ashbya gossypii* has been shown to be viable in the absence of a functional V-ATPase (38), and disruption of yeast genes encoding V-ATPase subunits resulted in only conditional lethality. Yeasts devoid of all V-ATPase activity are viable when grown in unbuffered media, grow optimally in media buffered to pH 5.5 (15), but fail to grow in alkaline-buffered media (pH > 7.5). Here we show that those growth conditions that favor viability of V-ATPase-deficient yeast also result in acidification of their vacuoles. When carried out in medium buffered to pH 5.5, measurements of vacuolar pH in Δuma mutants yielded an average value of 5.9 (Fig. 3). This suggests that acidification of the endosomal system, but not V-ATPase activity per se, is also indispensable in yeast.

Endocytosis Does Not Contribute to Vacuolar Acidification—
Acidic medium containing NH$_4^+$ right

...observed with the alkaline loading protocol (18, 35). Cell morphology (dye in the cytosol) were used for ratio measurements (1). The prediction orientation of the ATPase is such that Pma1p could assist in endosomal and/or vacuolar acidification.

Fig. 9. Effect of ammonium on the cytosolic pH of yeast. Wild-type (BJ926) yeasts and V-ATPase-deficient mutants (SF838-5A) were labeled with BCECF-AM under conditions favoring accumulation of the dye in the cytosol, as described in (18, 35). A, high magnification images showing cell morphology (top left) and the typical fluorescence staining observed with the alkaline loading protocol (top right). Intense cytosolic staining is apparent in three cells, with weak vacuolar staining present in the remaining cells. Cells with cytosolic staining were selected by setting an intensity threshold, and contaminating vacuoles were rejected by a size criterion >150 pixels (bottom left). The regions corresponding to the cytosol were used for ratio measurements (bottom right). B, images taken before and 90 min after exposure of cells to acidic medium containing NH$_4^+$. Bars, 5 μm. C, steady state cytosolic pH of wild-type (top) and Δuma yeasts (bottom), measured in media of pH 7.5 or 5.5 containing NH$_4^+$. An in situ calibration, obtained as in Fig. 2, was used to convert the fluorescence ratio into cytosolic pH values. D, time course of the cytosolic pH changes upon exposure to acidic medium containing NH$_4^+$. Cells were preincubated in pH 7.5 medium devoid of NH$_4^+$. The medium was then switched to pH 5.5 (white bar) and the cells subsequently challenged with Na$^+$ (solid squares) or NH$_4^+$ (open circles). Traces are representative of 3 separate experiments.

Assuming that vacuolar acidification is indispensable for survival, Nelson and Nelson (15) rationalized that V-ATPase-deficient yeasts must have alternative means of acidifying their vacuoles. Based on the observation that uma yeasts are only viable in acidic medium, they hypothesized that acid equivalents from the medium were internalized through fluid-phase endocytosis. A role for endocytosis in acidification was further supported by the observation that blocking endocytosis in a V-ATPase-deficient strain resulted in cell death (20). Endocytosis could also contribute to acidification by delivering the plasma membrane proton-pump ATPase, Pma1p, to the vacuole. The predicted orientation of the ATPase is such that Pma1p could assist in endosomal and/or vacuolar acidification.

We believe that this possibility is unlikely, as overexpression of Pma1p (which was confirmed by immunoblotting, Fig. 4) did not complement the Δuma phenotype (Table I) nor could any contribution to vacuolar acidification be detected by video microscopy (Fig. 5). Moreover, blocking endocytosis by introducing the temperature-sensitive mutation end4-1 did not prevent vacuolar acidification when the cells were suspended in acidic media. This was not due to a loss of the end phenotype at acidic pH, as uptake of Lucifer yellow remained strongly inhibited under these conditions. This observation not only rules out a role for endocytic delivery of Pma1p but also implies that vacuolar acidification does not result from uptake of the acidic fluid phase by pinocytosis, contrary to the original proposal of Nelson and Nelson (15).

Weak Acids in the Growth Media Can Contribute to Vacuolar Acidification—Our measurements of Δuma mutants bathed in minimal medium buffered to pH 5.5 yielded an average vacuolar pH of 5.9 (Fig. 3). This is seemingly contrary to previous reports that used quinacrine to demonstrate that Δuma mutant vacuoles were neutral (15, 25). However, this discrepancy can be readily explained by differences in the composition of the media used for the determinations. Quinacrine labeling was carried out in ammonium-containing rich media buffered to pH 7.5 (39). Imaging determinations performed in media of similar composition, and pH also resulted in a near-neutral pH value of 7.1 (Fig. 3).

Experiments such as those described above revealed that the vacuolar pH varies greatly and in some instances abruptly when the medium is replaced. What is the mechanism responsible for such changes? Alkalinization of the vacuole when substituting an acidic medium for a more alkaline one can be readily understood, considering that the growth media invariably contain high concentrations of ammonium (e.g. 38 mM). This cation is in equilibrium with the unprotonated form, ammonia, which readily permeates most biological membranes. After reaching the cytosol or the acidic vacuole, ammonia becomes protonated, thereby elevating the cytosolic or vacuolar pH (Fig. 10). That ammonia is responsible for the alkalinization was demonstrated by omitting ammonium from the medium. In this instance, raising the extracellular pH had little effect on either vacuolar pH or cytosolic pH over the period studied (Figs. 7 and 9).

The explanation for the abrupt acidification observed upon restoration of the acidic pH is less obvious. The rapidity of the effect suggests the entry and dissociation of a weak acid. On the other hand, perusal of the composition of the medium indicates that the major anions are all derived from strong acids (i.e. sulfate and chloride). Importantly, the acidification was observed only in media containing high concentrations of ammonium. At acidic pH, ammonium is predominantly in the protonated form, NH$_3^+$, because of its high pK$_a$ (9.26). The experiments of Fig. 8, where the cells were preincubated in the absence of NH$_3$/NH$_4^+$ ruled out the possibility that acidification...
is due simply to net efflux of the free base, NH₃. We therefore propose that NH₄⁺, a conjugated weak acid, mediates the delivery of acid equivalents into the vacuo (Fig. 10). Such an assumption implies that the plasma membrane possesses pathways for the permeation of the cationic species, NH₄⁺, which unlike lipophilic NH₃ cannot permeate the lipid bilayer. In this regard, yeasts have been shown to express at least two separate ammonium transport systems, Mep1p and Mep2p (reviewed in Refs. 40), on their plasmalemma. These transporters would deliver the conjugated weak acid to the cytoplasm where it deprotonates, thereby acidifying the cytosol (Fig. 10). Indeed, a rapid cytosolic acidification was observed upon exposure of cells to acidic medium containing NH₄⁺, but also to other weak acids present in the cytosol. Conversely, suspension of cells with acidic vacuoles in alkaline media will induce vacuolar alkalosis due to permeation and protonation of NH₃. The absence of NH₄⁺ in the medium used by Preston et al. (17) would explain why these authors found the vacuoles of V-ATPase-deficient yeast to be near neutral, pH 6.9.

In summary, we found that the vacuoles of yeasts incubated under acidic conditions that promote growth have a low pH and that vacuolar acidity, rather than H⁺ pumping per se, is essential for yeast viability. Moreover, we found that NH₃ and NH₄⁺, which are routinely added at high concentrations to growth media as a source of nitrogen, shuttle H⁺ equivalents across the plasma and/or vacuolar membranes. The former likely permeates the lipid bilayer, whereas the latter possibly utilizes specific transporters such as Mep1p and Mep2p or other transporters to deliver acid equivalents to the cytosol and to the vacuole.

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