Mutations of pma-1, the Gene Encoding the Plasma Membrane H⁺-ATPase of Neurospora crassa, Suppress Inhibition of Growth by Concanamycin A, a Specific Inhibitor of Vacular ATPases

(Received for publication, January 14, 1997)

Emma Jean Bowman, Forest J. O’Neill, and Barry J. Bowman

From the Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

Concanamycin A (CCA), a specific inhibitor of vacuolar ATPases, inhibited growth of Neurospora crassa in medium adjusted to pH 7 or above. Mutant strains were selected for growth on medium containing 1.0 μM CCA. Sixty-four (of 66) mutations mapped in the region of the pma1 locus, which encodes the plasma membrane H⁺-ATPase. Analysis of V-ATPase activity in isolated vacuolar membranes from the mutant strains showed wild-type activity and sensitivity to CCA. In contrast, plasma membrane H⁺-ATPase activity in isolated plasma membranes from the mutants was reduced as compared with wild-type, and in four strains the activity showed increased resistance to vanadate. The most interesting change in the plasma membrane H⁺-ATPase was in kinetic behavior. The wild-type enzyme showed sigmoid dependence on MgATP concentration with a Hill number of 2.0, while the seven mutants tested exhibited hyperbolic kinetics with a Hill number of 1.0. One interpretation of these data was that the enzyme had changed from a functional dimer to a functional monomer. Mutation of the plasma membrane H⁺-ATPase did not confer resistance by preventing uptake of CCA. In the presence of CCA both wild-type and mutant strains were unable to accumulate arginine, failed to concentrate chloroquine in acidic vesicles, and exhibited gross alterations in hyphal morphology, indicating that the CCA had entered the cells and inactivated the V-ATPase.

Concanamycin A (CCA)³ and bafilomycin A₁, members of a family of macrolide antibiotics, which we and our collaborators identified as specific and potent inhibitors of vacuolar H⁺-translocating ATPases (V-ATPases) (1, 2). Both compounds have subsequently been used in numerous in vivo studies with fungi, plants, and animals to implicate V-ATPases in a variety of biological processes, including bone resorption (3), toxin delivery (4), viral entry (5, 6), membrane targeting (7, 8), apoptosis (9, 10), regulation of cytoplasmic pH (11, 12), proteolytic processing (13), and acidification of intracellular systems (14–16).

Selection for resistance to antibiotics has been an important strategy for obtaining mutant forms of ion-translocating ATPases in fungal cells. For example, oligomycin allowed for selection of mutant forms of the mitochondrial ATPases of Neurospora crassa and Saccharomyces cerevisiae (17). Similarly, hygromycin B and Dio-9 were used to obtain mutants of the plasma membrane H⁺-translocating ATPases of S. cerevisiae and Schizosaccharomyces pombe (18–20). Because macrolide antibiotics are such good inhibitors of V-ATPases, we reasoned that they might enable us to obtain V-ATPase mutants in N. crassa, which would be valuable for investigating the mechanism of inhibition of the enzyme by the antibiotic and in defining the role of the vacuolar ATPase in the cell.

V-ATPases are large, complex enzymes located on the membranes of acidic compartments within all eukaryotic cells and on plasma membranes of some specialized cells (21). Their many subunits are arranged into a hydrophilic, dissociable part (13), and acidification of intracellular systems (4), viral entry (5, 6), membrane targeting (7, 8), apoptosis (9, 10), regulation of cytoplasmic pH (11, 12), proteolytic processing (13), and acidification of intracellular systems (14–16).

Little is known about the mechanism by which concanamycins and bafilomycins inhibit V-ATPases. Bafilomycin A₁ has been shown to interact with the membrane region of the bovine coated vesicle V-ATPase, and reconstitution experiments suggest that the binding site of bafilomycin A₁ may be the 100-kDa subunit (25, 26). By selecting for mutant strains that are resistant to CCA, we thought it might be possible to generate mutants altered in the CCA binding site.

Although we expected to obtain mutations in V-ATPase genes, instead, as will be shown below, we isolated mutations in the gene encoding the plasma membrane H⁺-ATPase. This enzyme, a P-type ATPase, is completely different from the V-ATPase in structure and mechanism (reviewed in Ref. 27). It consists of a single type of large subunit and undergoes phosphorylation as part of its reaction cycle. We want to emphasize that the plasma membrane H⁺-ATPase of N. crassa was not responsible for the inhibition of growth by CCA. Instead, we hypothesize that the mutations conferred resistance by preventing the entrance of CCA into the cells.
Concanamycin-resistant Mutants of N. crassa

inhibited by bafilomycin A\textsubscript{1}, even at micromolar concentrations that did inhibit P-ATPases (Na\textsuperscript{+}/K\textsuperscript{+} and Ca\textsuperscript{2+}) of mammalian cells (1). As mentioned above, hygromycin B, which does not directly affect the plasma membrane H\textsuperscript{+}-ATPase of S. cerevisiae, was successfully used to isolate plasma membrane H\textsuperscript{+}-ATPase mutants in this organism. It was suggested that resistance to hygromycin B was conferred because the cells were no longer able to take up the antibiotic. Alternatively, because in the CCA-resistant strains of N. crassa resistance to hygromycin B was conferred because the cells were ATPase mutants in this organism. It was suggested that resistance to CCA might also act by preventing uptake of the antibiotic. Alternatively, because in the CCA-resistant strains of N. crassa resistance to hygromycin B was conferred because the cells were ATPase mutants in this organism. It was suggested that resistance to CCA might also act by preventing uptake of the antibiotic.

In the present report we examined the effect of CCA on growth of N. crassa and investigated the properties of antibiotic-resistant mutants. We found that mutations of the plasma membrane H\textsuperscript{+}-ATPase conferred resistance but did not restrict uptake of CCA. Thus, the plasma membrane H\textsuperscript{+}-ATPase must act in some other way than preventing uptake to suppress the inhibition of growth caused by the antibiotic.

**EXPERIMENTAL PROCEDURES**

**Isolation and Mapping of CCA-resistant Mutants—**Conidia (asexual spores) from wild-type strain 74A were mutagenized with UV light and spread on agar plates containing Vogel’s medium N (29), 2.0% sucrose, 20 mM HEPES, and 1.0 \( \mu \)M CCA, adjusted to pH 7.2 with NaOH. CCA, dissolved in dimethyl sulfoxide (Me2SO), was added after autoclaving. Control media contained an equivalent volume of Me2SO, which had no detectable effect on growth. Wild-type conidia did not grow on this medium. After 3 to 5 days at 30 °C, colonies that could grow were picked and transferred to tubes with 1 ml of Vogel’s medium N with 2% sucrose. The putative resistant strains were backcrossed to the isogenic strain 74a, and progeny were screened for resistance to CCA by spotting on medium adjusted to pH 7.2 and containing 0.2 \( \mu \)M CCA. Strains were named ccr for concanamycin A resistant.

The chromosomal location of the mutations was mapped by standard techniques (30). The mutation in 10 CCA-resistant isolates was first mapped to LGII using the alcoy marker strain. The isolates were then crossed with strains mutated in thr-2, arg-5, or arg-12 to locate the locus more closely to LGIII near arg-12 (31). Fifty-six additional ccr strains were crossed with cer-11a, and 40–50 progeny were isolated from each cross. For 54 of the ccr strains all progeny were resistant, suggesting that the mutations mapped to the same locus as in cer-11a. In two isolates wild-type progeny were recovered. The mutations in these 2 isolates were subsequently mapped to LGII and LGVII and will not be considered further here. We did not undertake complementation analysis, because it would require forced heterokaryons, which would have variable nuclear ratios, making the results difficult to interpret for an antibiotic-resistant phenotype.

**Growth Tests—**Spot tests were employed to test for resistance to CCA or hygromycin B. Typically, isolated spores from a cross were allowed to grow in 1 ml of liquid Vogel’s medium N supplemented with 2% sucrose until plentiful conidia were produced (4–5 days at 30 °C and 1–5 more days at room temperature) and tested for growth by spotting a loopful of vortexed conidial suspension on agar plates. Resistance to CCA was scored on the pH 7.2 medium described above after 1 day of growth at 30 °C in the absence of CCA or after 2 days in the presence of 0.2 \( \mu \)M CCA. Resistance to hygromycin B was scored after 2–4 days of growth on media containing Vogel’s medium N supplemented with 2% sorbose (to induce colonial growth), 0.05% fructose, 0.05% glucose, and 0.025% inositol in the absence or presence of hygromycin B (0–200 \( \mu \)g/ml) added before autoclaving. Strain FO13, which was transformed with a plasmid encoding hygromycin phosphotransferase (pCSN433) (32), served as a positive control for resistance to hygromycin B.

For quantitative comparisons of the growth rates of wild-type and ccr strains, we measured colony diameters on agar plates containing Vogel’s medium N supplemented with 2% sucrose and 1.5 M KCl or 0.2 M ammonium acetate. Agar plates at pH 5.8 (unadjusted) or adjusted to pH 7.2 (containing 20 mM HEPES and NaOH) and supplemented with 0–10.0 \( \mu \)M CCA were also used for growth rate tests. In these tests plates were inoculated with conidia in 3 \( \mu \)l of water/plate. Colony diameter was measured at 24-h intervals during growth at 30 °C.

**Preparation of Membranes and Assays of ATPase Activity—**Plasma membranes, mitochondria, and vacuolar membranes were isolated from wild-type and mutant strains by the bead-beater procedure described by Bowman and Bowman (33) with two minor changes. We included 1 mM phenylmethylsulfonyl fluoride in the buffer used to break the cells, and we separated mitochondria and vacuoles from each other by centrifugation through a step gradient, containing 7 mM of 50% (v/v) of 20% sucrose solution. ATPase activities were assayed at 30 °C by the release of inorganic phosphate as described (33). Reaction mixtures for mitochondrial and V-ATPases were as described previously except that MgCl\(_2\) was replaced with MgSO\(_4\). Plasma membrane H\textsuperscript{+}-ATPase activity was assayed with 5–15 \( \mu \)g of membrane protein in a reaction mixture (0.5 ml) containing 5 mM \( \mathrm{Na}_2\)ATP, 5 mM MgSO\(_4\), 15 mM NH\(_4\)Cl, 3 mM phosphoenolpyruvate (manganese salt), 25 \( \mu \)g of pyruvate kinase, 5 mM K\textsubscript{NAD}, (to inhibit residual mitochondrial ATPase), 0.1 \( \mu \)M CCA (to inhibit residual V-ATPase), and 10 mM Pipes buffer, adjusted to pH 6.7 with Tris base. After learning that the plasma membrane H\textsuperscript{+}-ATPase activity in ccr strains was somewhat unstable, we carried out all assays of this enzyme for 10-min periods using a freshly thawed and diluted aliquot of plasma membranes. To determine the Km of the plasma membrane H\textsuperscript{+}-ATPase for its substrate, MgATP, we added equal concentrations of MgSO\(_4\) and Na\textsubscript{ATP} (adjusted to pH 6.7 with Tris base) to give a final concentration ranging from 0.05 to 7.0 mM of each. For determinations of specific activity, protein was assayed by the method of Lowry et al. (34) with bovine serum albumin as standard. Data from each enzyme assay were plotted and analyzed with the aid of the KaleidaGraph program.

**Arginine Content—**Twenty-five-ml volumes of liquid medium in 125-ml Erlenmeyer flasks were inoculated with 1 \( \times \) 10\(^6\) conidia/ml and grown on a reciprocating shaker for 48 h at 25 °C. The four types of media consisted of Vogel’s medium N with 2% sucrose supplemented with 1) no additions; 2) 0.2 \( \mu \)M CCA; 3) 20 mM HEPES, pH to 7.2 with NaOH; and 4) 20 mM HEPES, pH to 7.2 with NaOH, and 0.2 \( \mu \)M CCA. Cells were harvested by filtration on Whatman 541 paper, rinsed with distilled H\(_2\)O, frozen in liquid nitrogen, and lyophilized. The dried material was weighed to assess growth yield, ground up with a small plastic pestle, and aliquoted for arginine determinations. 10-mg aliquots of lyophilized cells were extracted with 5% trichloroacetic acid for 20 min on ice and centrifuged 10 min at 10,000 rpm in a refrigerated tabletop microcentrifuge to release acid-soluble arginine. Suitable volumes of supernatant were used for determinations of arginine content by the Sakaguchi method (35) with 0–100 nmol of arginine as standard. Arginine content in wild-type and ccr mutants grown in the different media was compared as nanomole of Arg/mg dry weight.

**Microscopy—**Cultures were grown as colonies on agar plates. Regions of the growing front were photographed directly with Kodak print film, ASA 100, using a Leitz Aristoplan microscope and camera. Figures were assembled using the program Photoshop.

**Materials—**The CCA used in these experiments was isolated in the laboratory of Dr. Axel Zeeck, University of Göttingen. CCA is commercially available from Fluka Chemical Corp. (Ronkonkoma, NY) and Wako Chemicals Inc. (Richmond, VA).

**RESULTS**

**Isolation of Mutants—**Both concanamycins and bafilomycins have been shown to be potent inhibitors of V-ATPase activity at nanomolar concentrations. We used CCA for this study because it is easier to isolate and more stable than the previously used bafilomycin A\textsubscript{1} (2). Because bafilomycin A\textsubscript{1} inhibited some P-ATPases from mammalian cells at micromolar concentrations (1), we first compared the effect of CCA on the three H\textsuperscript{+}-ATPases of N. crassa. The V-ATPase was highly sensitive to CCA, with a \( K_c \) of 3 nm, and both the plasma membrane and mitochondrial (F-type) ATPases were resistant at concentrations up to 10 \( \mu \)M (Fig. 1).

In preliminary growth tests, we found conditions under which CCA prevented growth of the wild-type strain, 74A (see “Experimental Procedures”). One important variable was the pH of the medium. At pH 5.8, the fluorescent pH of Vogel’s minimal medium, growth of conidia on agar plates was slowed by CCA but not prevented. Increasing the pH of the medium to neutral or higher pH resulted in prevention of growth by CCA. A second important variable was the carbon source in the medium. Typically, N. crassa mutants are selected on medium containing sorbose as a carbon source, which confers colonial growth to the

Downloaded from http://www.jbc.org/ by guest on July 25, 2018

17477
mycelial fungus. However, inhibition of growth by CCA was more consistent and reproducible in strains grown on sucrose, the usual carbon source in Vogel’s minimal medium.

Under the selective conditions described under “Experimental Procedures,” 66 CCA-resistant mutants were isolated. Most of them fell into two phenotypic groups: 1) short and strongly resistant or 2) tall and weakly resistant. Short versus tall refers to the height of aerial hyphae and conidia above the surface in 10 × 75-mm test tubes containing 1 ml of liquid medium. Short strains typically grew 12–14 mm above the liquid surface. Tall strains showed more variability, growing from 15 to 39 mm above the surface at the extremes, but most often they were 15–25 mm above the liquid surface. Strongly versus weakly resistant refers to the size of colony produced on the CCA spot tests, included in Table I. Wild-type was tall and not resistant. In genetic crosses 64 of the mutants mapped close to the arg-12 locus on LGHII; however, pma-1, which encodes the plasma membrane H^+-ATPase of N. crassa, has been localized here by restriction fragment length polymorphism mapping (36).

Thinking that the N. crassa CCA-resistant mutants might resemble the hygromycin B-resistant pma1 mutants in S. cerevisiae (18), we screened for phenotypic similarities by performing spot tests with 50 of the N. crassa mutants. Unlike the yeast mutants, the N. crassa strains were not particularly sensitive to either high or low pH, although they did grow less well at all pH values than the wild-type strain; none were resistant to hygromycin B or cycloheximide; none were inhibited by growth on 0.2 M ammonium sulfate; and none were cold or temperature sensitive when compared with the wild-type strain. However, like the pma1 mutants of yeast, our ccr mutant strains were more sensitive to high ionic or osmotic pressure conditions than the wild-type (shown below).

Seven CCA-resistant strains, 5 short (ccr1-15a, ccr4-5A, ccr40-8A, ccr15-8-6-40A, and ccr6-25a) and 2 tall (ccr18-1a and ccr21-2a), were chosen for more detailed characterization. Growth rates of wild-type (74A) and mutant strains on 6 different solid media were compared (Table I). Note that strains were allowed to grow for different lengths of time on different media to have larger colony diameters for measurement and comparison. At pH 7.2 in the absence of CCA, 4 short mutants (ccr1-15a, ccr4-5A, ccr40-8A, and ccr6-25a) and 1 tall mutant (ccr21-2a) grew nearly as well as 74A, while 1 short mutant (ccr15-8-6-40A) and 1 tall mutant (ccr18-1a) grew more slowly. At pH 7.2 in the presence of CCA all of the short mutants, including ccr15-8-6-40A, appeared to be more strongly resistant, i.e. attained a larger diameter than the tall mutants; 74A did not grow. At pH 5.8 in the absence of CCA, the standard Vogel’s minimal medium for growth of N. crassa, the mutants grew somewhat more slowly than 74A. At pH 5.8 in the presence of CCA 74A was able to grow, and all the mutant strains grew as well or better than the wild-type. Although strains might grow in the presence of CCA, in all cases growth on the antibiotic was restricted as compared with in its absence, resulting in a colonial phenotype.

If the CCA mutations were indeed in the plasma membrane H^+-ATPase, as suggested by the mapping results, the mutant strains might be defective in the ability to regulate cytoplasmic ion concentration or pH and therefore grow poorly on media with high concentrations of potassium chloride or acetate as described for the pma1 mutant strains of S. cerevisiae (18). This proved to be the case. On minimal medium supplemented with either 0.2 M CH_3COONH_4 or 1.5 M KCl, the mutant strains grew significantly less than the wild-type strain (Table I). Four strains grew 30–60% as well as 74A on 0.2 M CH_3COONH_4, while three strains were particularly impaired, growing less than 20% as fast as 74A. Five mutant strains grew about 30–50% as fast as 74A on 1.5 M KCl, while two grew considerably faster (about 80% as fast as 74A). These results were consistent with the possibility that the mutations might be in the plasma membrane H^+-ATPase. Furthermore, the results in Table I indicated that different CCA-resistant isolates had different phenotypes and were unlikely to be simple “loss of function” mutants.

Because the CCA-resistant mutants showed some pheno-

| Table I | Effect of pH, CCA, ammonium acetate, and high salt concentration on growth of wild-type and mutant strains |
|---------|---------------------------------------------------------------|
| Strain  | pH 7.2 (44 h)      | pH 7.2 + CCA (72 h)      | pH 5.8 (24 h)      | pH 5.8 + CCA (48 h)      | 0.2 M CH_3COONH_4 (72 h)      | 1.5 M KCl (96 h) |
| 74A     | 76                  | 0                     | 62                  | 44                  | 82                   | 40                   |
| ccr1-15a | 69                  | 37                    | 48                  | 58                  | 48                   | 16                   |
| ccr4-5A | 64                  | 35                    | 51                  | 53                  | 29                   | 13                   |
| ccr40-8A | 53                 | 33                    | 42                  | 52                  | 33                   | 15                   |
| ccr15-8-6-40A | 36             | 29                    | 44                  | 53                  | 10                   | 17                   |
| ccr6-25a | 53                 | 34                    | 61                  | 46                  | 13                   | 20                   |
| ccr18-1a | 35                 | 10                    | 55                  | 50                  | 34                   | 34                   |
| ccr21-2a | 53                 | 15                    | 56                  | 41                  | 14                   | 31                   |
Concanamycin-resistant Mutants of N. crassa

Conidial characteristics described for hygromycin B-resistant mutants in yeast (18), we tested the group of seven mutants more carefully for resistance to hygromycin B (0, 5, 20, 50, and 200 μg/ml). All seven mutants responded to hygromycin identically to the wild-type, with detectable inhibition at 20 μg/ml hygromycin and almost complete inhibition at 200 μg/ml (Table II). The positive control, strain FO13, carried a plasmid with a hygromycin B-resistance gene and grew equally well on media containing 0–200 μg/ml hygromycin.

**Evidence That the CCA Mutants Are Altered in the pmA1 Gene**—The initial characterization of the CCA-resistant mutants suggested that they were altered in the gene encoding the plasma membrane H^+-ATPase. To test this prediction, plasma membranes were isolated, and ATPase activity was assayed. Plasma membrane H^+-ATPase activity in mutant strains was consistently lower than that of the wild-type strain as shown in Table III. The specific activity of ATPase in plasma membrane fractions isolated from mutant strains ranged from 17 to 84% of the average value obtained for the wild-type strain. Two caveats apply to these results. First, we saw significant variability in plasma membrane H^+-ATPase specific activities: for example, values for 74A ranged from 2.6 to 4.7 μmol/min/mg in five experiments. Second, the enzyme from the mutant strains was unstable, either in ice or at 30 °C while being assayed. To compensate for the instability, we froze multiple aliquots of plasma membranes, thawed an aliquot just before assay, and limited the assay time to 10 min.

Vanadate is a potent inhibitor of P-ATPases (27). The plasma membrane H^+-ATPase of some of the CCA-resistant mutants was resistant to inhibition by vanadate. For example, half-maximal inhibition by vanadate was reached at 1.0 μM for the wild-type enzyme and at 23.4 μM for the plasma membrane H^+-ATPase of ccr40-8A (Fig. 2). Data from the other mutants examined are summarized in Table III. Among the 7 mutants tested, 4 displayed increased resistance to vanadate, and 3 behaved like the wild-type. The vanadate-resistant strains were all short and strongly resistant in phenotype, while the tall, weakly resistant strains exhibited normal sensitivity to vanadate. One mutant, ccr6-25a, did not fall into either group, because it was short and strongly resistant to CCA but not resistant to vanadate.

The most striking change in the plasma membrane H^+-ATPase from the mutants was its kinetic behavior. A plot of plasma membrane H^+-ATPase activity versus substrate (MgATP) concentration with the enzyme from 74A showed distinctly sigmoid kinetics (Fig. 3A), previously interpreted as indicative of two interacting substrate-binding sites (37). In sharp contrast, the curve for the plasma membrane H^+-ATPase from ccr40-8A was hyperbolic, suggestive of a single substrate-binding site. Further analysis of these data by the Hill plot generated a straight line with a slope of 2.2 for the wild-type and with a slope of 1.0 for the mutant plasma membrane H^+-ATPase (Fig. 3B). This characteristic was shared by all seven CCA-resistant mutants examined: their plasma membrane H^+-ATPase exhibited hyperbolic kinetics with activity plotted as a function of substrate concentration and gave a slope of approximately 1.0 on a Hill plot (Table III). A consequence of the change from sigmoid to hyperbolic kinetics was a lowering of the apparent K_m for substrate. The plasma membrane H^+-ATPase from 74A hydrolyzed MgATP with a half-maximal value of 1.2 mM, and values for the mutant enzymes ranged from 0.18 to 0.60 mM (Table III).

Vacuolar membranes were isolated from the seven ccr strains and assayed for V-ATPase activity. The specific activity of V-ATPase in the mutants was similar to the wild-type value, and the enzyme showed wild-type sensitivity to CCA (data not shown).

**Effect of CCA on Function of V-ATPase in ccr Strains**—In S. cerevisiae resistance to hygromycin appears to result from a failure of the cells to take up the antibiotic due to a change in the properties of the plasma membrane H^+-ATPase (28). We anticipated that a similar mechanism might explain the resistance of ccr mutants in N. crassa to CCA. However, several kinds of experimental data suggested this was not the case. Instead, CCA appeared to enter both mutant and wild-type cells and to inhibit the vacuolar ATPase.

An early indication that CCA was taken up by the ccr mutants came from the spot tests. CCA-resistant cells could grow on medium containing CCA but not nearly so well as on medium lacking CCA (Table I). Even with sucrose as the carbon source, the resistant strains had a partially colonial growth morphology on medium containing CCA, suggesting that the inhibitor was getting into the cells and exerting an

---

**Table II**

| Strain | Relative growth on hygromycin B |
|--------|--------------------------------|
|        | 0 μg/ml | 20 μg/ml | 200 μg/ml |
| FO13   | ++++++   | ++++++   | ++++++    |
| 74A    | ++++++   | ++++    | +         |
| ccr1–15a | ++++   | ++++      | +         |
| ccr4–5A | ++++   | ++++      | +         |
| ccr40–8A | ++++   | ++++      | +         |
| ccr57–4a | ++++   | ++++      | +         |
| ccr6–25a | ++++   | ++++      | +         |
| ccr18–1a | ++++   | ++++      | +         |
| ccr21–2a | ++++   | ++++      | +         |

**Table III**

| Strain     | PM ATPase activity | K_i for vanadate | K_m for MgATP | Hill plot number |
|------------|--------------------|------------------|---------------|-----------------|
| 74A (wild-type) | 3.2 | 1.0 | 1.20 | 2.23 |
| ccr1–15a | 1.3 | 16.4 | 0.22 | 1.15 |
| ccr4–5A | 1.8 | 19.0 | 0.18 | 0.90 |
| ccr40–8A | 2.3 | 23.4 | 0.26 | 1.03 |
| ccr15–8–6–40A | 2.7 | 14.0 | 0.30 | 1.40 |
| ccr6–25a | 1.1 | 1.0 | 0.60 | 1.22 |
| ccr18–1a | 2.6 | 0.7 | 0.34 | 1.22 |
| ccr21–2a | 0.55 | 2.0 | 0.33 | 1.03 |

Note: K_i = 0.5 mM for substrate-binding site. The plasma membrane H^+-ATPase from 74A hydrolyzed MgATP with a half-maximal value of 1.2 mM, and values for the mutant enzymes ranged from 0.18 to 0.60 mM (Table III).

---

* n is the Hill number, indicating the apparent degree of cooperativity between substrate-binding sites.
Concanamycin-resistant Mutants of N. crassa

FIG. 2. Effect of vanadate on plasma membrane H^+-ATPase activity in wild-type and mutant strains. Plasma membranes were isolated and ATPase activity determined as described under "Experimental Procedures." Control activities were 3.0 μmol/min/mg protein for 74A (wild-type) and 1.9 μmol/min/mg protein for ccr40-8A (CCA-resistant strain). Apparent K_m values for vanadate were 1.0 μM for 74A and 23.4 μM for ccr40-8A. Results from other ccr strains are summarized in Table III.

effect. In analyses of growth rate (measured as colony diameter) versus CCA concentration, the wild-type and mutant strain ccr1-15a responded similarly (Fig. 4). Both grew at CCA concentrations up to 0.001 μM CCA, both were inhibited about 50% at 0.01 μM CCA, and both were maximally inhibited at concentrations of 0.1 to 10.0 μM. The difference was that the wild-type did not grow at all at the higher concentrations, but the mutant continued to grow at about the same reduced rate when the CCA concentration was increased by 3 orders of magnitude (Fig. 4).

To assess the effect of CCA on activity of the vacuolar ATPase in vivo we decided to use whole cell arginine content as an indicator. Arginine, together with other basic amino acids, is stored at high concentrations in fungal vacuoles (38, 39). Accumulation of arginine in the vacuole is dependent on the proton gradient generated by the V-ATPase (40). We reasoned that if the V-ATPase were inactivated by CCA added to the medium, we should see greatly reduced levels of arginine in whole cell extracts. Wild-type and ccr mutants were grown in shaking liquid cultures at pH 5.8 and 7.2 in the presence and absence of 1.0 μM CCA. Growth yield and arginine content were determined (Table IV). Strain 74A grew about equally well at pH 5.8 and 7.2. At pH 5.8, cells accumulated 35 nmol of arginine/mg dry weight. At pH 7.2, they had 34% lower levels. As mentioned above, growth of 74A in the presence of CCA was distinctly pH-sensitive. At pH 5.8, growth was inhibited 57% by CCA, but at pH 7.2 it was stopped. (The small amount of growth seen in the liquid culture at pH 7.2 occurred only at the meniscus of the sloshing culture.) Addition of CCA to the wild-type cultures had an identical effect at both pH 5.8 and 7.2 on arginine content, which was reduced to a basal level of approximately 5 nmol/mg dry weight. The most plausible explanation for these results was that CCA entered the cells, inactivated the V-ATPase, and prevented accumulation of arginine in the vacuole.

In the absence of CCA, 5 of the mutant strains, classified as short and strongly CCA resistant, grew much like the wild-type, with similar growth yields at both pH 5.8 and 7.2 (Table IV). The two mutant strains classified as tall and weakly CCA-resistant displayed pH-sensitive growth, growing about one-half as much at pH 7.2 as at pH 5.8. Like the wild-type cells, addition of CCA to the medium at pH 5.8 partially inhibited growth of all the mutants; however, unlike wild-type, all the mutant strains grew significantly at pH 7.2, with growth yields of 24–75% of that observed in the absence of CCA. With respect to their arginine content, the mutant strains behaved like the wild-type in all four conditions. Accumulation of arginine in the absence of CCA was pH-dependent; less accumulated at the higher pH. Addition of CCA also had a common effect. In all cases, the arginine level was reduced to a basal level ranging from 2.3 to 4.9 nmol of arginine/mg dry weight. Because CCA was equally effective in preventing arginine accumulation in mutant cells and wild-type cells, it would appear that CCA did enter the ccr mutants and did inhibit the vacuolar ATPase. Nonetheless, the mutant cells were able to grow at pH 7.2 in the presence of CCA.

An alternative method for looking at activity of the V-ATPase in vivo is to test for acidification of the vacuolar interior by using dyes that accumulate and fluoresce in acidic environments. When 74A cells of N. crassa are incubated with the weak base chloroquine, many small brightly staining organelles, believed to be vacuoles, are visible by fluorescent microscopy (41). Incubation of 74A cells with CCA prevented the appearance of the brightly fluorescing bodies, indicating that the vacuoles could not be acidified. Similar results were obtained with the 7 CCA-resistant mutants characterized in this paper. In a separate series of experiments we grew wild-type and ccr1-15a mutant cells in aerated cultures at pH 5.8 and 7.2 in the presence and absence of 1.0 μM CCA. Again, both types of cells grown in the absence of CCA accumulated the fluorescent dye, and cells grown in the presence of CCA did not.

FIG. 3. Dependence of ATPase activity on substrate concentration in wild-type and mutant strains. Plasma membranes were isolated and ATPase activity assayed as described under “Experimental Procedures.” A, velocity (specific activity) versus substrate (MgATP) concentration showing a sigmoid response for the wild-type (74A) and a hyperbolic response for the mutant (ccr40-8A). B, Hill analysis of the data used in A resulting in a Hill number of 2.23 for the wild-type (74A) and 1.03 for the mutant (ccr40-8A). V_max was 43.5 nmol/min for 74A and 16.0 nmol/min for ccr40-8A.
Thus, both wild-type and ccr mutant cells incubated in CCA failed to accumulate chloroquine in their vacuoles, indicating that the vacuoles had not been acidified (data not shown).

The inhibitory effect of CCA might be primarily on germination of conidia rather than on growth of vegetative mycelia. To test this possibility we allowed conidia from 74A and ccr1-15a to germinate and grow 24 h before adding the inhibitor. We then determined the increase in dry weight between 24 h and 48 h and found that the wild-type, as well as the mutant strain, grew almost as well in the presence of CCA as in its absence at pH 5.8 (Table V). At pH 7.2 the addition of CCA slowed the growth of wild-type about 25% and had little effect on the mutant. The arginine content in the samples grown in the presence of CCA was between 5 and 7 nmol/mg dry weight, slightly higher than in cultures where CCA was added before inoculation with conidia (Tables IV and V).

N. crassa acidifies the medium during growth. Perhaps 74A could grow vegetatively in the pH 7.2 medium with CCA added at 24 h because sufficient growth had taken place to lower the pH of the medium to below 7.0. Indeed, for the 74A culture in the experiment of Table V, the pH of the medium was 6.9 after 24 h of growth. To determine if 74A could grow in the presence of CCA at a basic pH we used medium with a higher pH and with a higher concentration of buffer (100 mM HEPES, pH adjusted to pH 7.7 with NaOH) (Table VI). Again, CCA significantly inhibited growth, by about 60%, but it did not completely prevent vegetative growth even though the pH of the medium was maintained at pH 7.3 or higher. Overall, these results indicated that CCA had a more pronounced effect on germination of spores than on vegetative growth of mycelia, preventing germination of conidia (or ascospores, data not shown) at alkaline pH and slowing, but not stopping, growth of hyphae.

Effect of CCA on Hyphal Morphology—While investigating the properties of the ccr mutants we observed that CCA had a striking effect on hyphal morphology. Normal hyphal morphology is illustrated in Fig. 5 by strain 74A grown on agar plates for 1 day at pH 5.8. N. crassa grows by apical extension of hyphae, with branches occurring most often subapically at regularly spaced intervals. Addition of CCA to the medium caused a dramatic change in the growth pattern (Fig. 5). The growth rate (colony diameter) was substantially reduced (Table I), and the mycelium lost its relatively homogeneous appearance. Polarized tip growth in particular seemed disrupted with multiple apices formed at most branch points. Although profuse branching was most common, some regions showed long extensions of hyphae before branching occurred. At pH 5.8 the mutant strain ccr40-8A looked similar to the wild-type both in

![Graph of Colony Diameter vs. Concentration of Concanamycin A](image.png)

**TABLE IV**

| Strain     | pH 5.8 -CCA mg | +CCA mg | pH 7.2 -CCA mg | +CCA mg | Arginine content pH 5.8 nmol/mg | +CCA nmol/mg | pH 7.2 -CCA nmol/mg | +CCA nmol/mg |
|------------|----------------|---------|----------------|---------|--------------------------------|--------------|---------------------|--------------|
| 74A        | 141            | 61      | 141            | 2       | 34.6                           | 5.3          | 23.0                | 4.9          |
| ccr1-15a   | 108            | 82      | 131            | 95      | 28.5                           | 3.1          | 18.8                | 3.0          |
| ccr4-5A    | 141            | 125     | 128            | 80      | 29.4                           | 4.0          | 12.0                | 4.5          |
| ccr40-8A   | 157            | 95      | 158            | 119     | 26.5                           | 3.2          | 9.6                 | 3.9          |
| ccr15-8-6-40A | 123    | 80      | 130            | 55      | 35.4                           | 4.9          | 13.6                | 4.7          |
| ccr6-25a   | 135            | 95      | 137            | 24      | 35.8                           | 3.5          | 9.0                 | 3.3          |
| ccr18-1a   | 90             | 29      | 55             | 13      | 17.8                           | 2.3          | 11.8                | 2.6          |
| ccr21-2a   | 99             | 42      | 45             | 23      | 18.8                           | 2.7          | 8.9                 | 2.3          |

**TABLE V**

| Strain     | pH 5.8 dry weight at 24 h mg | pH 7.2 dry weight at 24 h mg | pH 5.8 Increase in dry weight between 24 and 48 h mg | pH 7.2 Increase in dry weight between 24 and 48 h mg | Arginine content pH 5.8 nmol/mg | +CCA nmol/mg | pH 7.2 +CCA nmol/mg |
|------------|-----------------------------|-----------------------------|----------------------------------------------------|----------------------------------------------------|--------------------------------|--------------|---------------------|
| 74A        | 23                          | 22                          | 125                                                | 114                                                | 125                            | 94           | 38.8                | 5.6          |
| (pH)       | (5.3)                        | (6.9)                       | (4.1)                                              | (3.9)                                              | (6.3)                           | (6.3)        | 23.2                | 7.0          |
| ccr1-15a   | 19                          | 24                          | 107                                                | 117                                                | 121                            | 116          | 27.0                | 4.7          |
| (pH)       | (5.4)                        | (6.9)                       | (4.6)                                              | (4.3)                                              | (6.5)                           | (6.5)        | 15.2                | 6.0          |
the absence and presence of CCA (Fig. 5). In general CCA had
a similar effect on the morphology of the other ccr strains (data
not shown).

Raising the pH of the medium to 7.2 affected growth of the
wild-type culture primarily by slowing the rate of hyphal ex-
tension at the apex (Fig. 6 and Table I). Branching continued
to occur laterally. Overall the branching pattern resembled the
pattern at pH 5.8 except that branch points occurred at shorter
intervals as a consequence of the reduced rate of hyphal elon-
gation. In the presence of CCA at pH 7.2 the wild-type conidia
did not germinate, and no growth occurred (Fig. 6). At pH 7.2 in
the absence of CCA the mutant cells resembled the wild-type
strain in their growth habit. At pH 7.2 in the presence of CCA,
the mutant strains could grow, but their morphology was se-
verely affected by the inhibitor (ccr40-8A, Fig. 6; other ccr
strains, data not shown). The appearance of the mycelium was
an exaggerated version of that seen at pH 5.8. Apical extension
of the hyphae was more inhibited; therefore, the colony ex-
tended more slowly. Again, branching was profuse at apices
and highly irregular. After 2 days of growth the defects in the
mutants were even more pronounced. Four examples are illus-
trated in Fig. 7.

DISCUSSION

Selection for Resistance to CCA Yields Strains with Mutations in the Plasma Membrane H⁺-ATPase—In this paper we
have described a procedure for selecting strains of N. crassa
that are resistant to CCA, a specific and potent inhibitor of
V-ATPases (2). To our surprise 64 of 66 independent isolates
appear to be mutated in the pma1 gene, which encodes the
plasma membrane H⁺-ATPase (42, 43). Null mutations in this
gene are likely to be lethal, as has been demonstrated for the
PMA1 gene of S. cerevisiae (44). The seven ccr strains examined
in detail were found to have reduced, but significant levels of
plasma membrane H⁺-ATPase activity, measured both by ATP
hydrolysis (Table III) and by proton pumping. The standard
growth medium the mutant strains grew as well as wild-type or
at modestly reduced rates. However, under conditions which
might require full activity of the plasma membrane H⁺-
ATPase, such as growth on 0.2 M ammonium acetate or 1.5 M
KCl, all of the mutants grew considerably less than the wild-
type (Table I).

The most interesting characteristic of the ccr strains was a
distinct change in the kinetic behavior of the plasma mem-
brane H⁺-ATPase. In the wild-type, the rate of ATP hydrolysis
showed a sigmoid dependence on MgATP concentration. A Hill
plot of the data had a slope of approximately 2, suggesting

| Additions | Growth yield | pH of medium |
|-----------|--------------|--------------|
| Me₂SO, 0 h | 141 | 7.3 |
| CCA, 0 h | 0 | 7.7 |
| Me₂SO, 24 h | 127 | 7.3 |
| CCA, 24 h | 51 | 7.3 |

2 E. J. Bowman, F. J. O’Neill, and B. J. Bowman, unpublished results.
positive cooperativity between two substrate-binding sites. In all 7 ccr strains examined in detail, the kinetics fit well to the Michaelis-Menten equation, and the Hill plot showed a slope of approximately 1 (Fig. 3, Table III).

We have previously suggested that in *N. crassa* the plasma membrane H⁺-ATPase functions as a dimer with positive cooperativity between active sites (37). In addition to the analysis of the rates of MgATP hydrolysis, other data support this conclusion. Vanadate, a potent inhibitor of the enzyme when assayed at high MgATP concentrations, can activate the enzyme and stimulate activity at low MgATP concentrations (45). Data from radiation inactivation experiments showed a functional target size of 230 kDa, close to the predicted value for two 100-kDa subunits (46). Similar kinetic data, consistent with positive cooperativity, have been obtained with purified plasma membrane H⁺-ATPase from *S. cerevisiae* (47). Interestingly, that study reported that the sigmoid kinetics were not so obvious in assays of plasma membranes as in assays of the purified enzyme. (In *N. crassa* both membranes and purified enzyme exhibit pronounced sigmoid kinetic behavior.) Genetic data strongly suggest that the plasma membrane H⁺-ATPase from *S. cerevisiae* exhibit allelic complementation; two *pma1* alleles that individually give rise to mutant phenotypes will confer a wild-type phenotype when expressed together in the same cell (18).

However, the oligomeric structure of the enzyme and the role of site-site interaction in the mechanism is still unresolved. The kinetic data can be fit to alternative models, e.g., a monomeric enzyme with two catalytically active conformations. Experiments with the *N. crassa* plasma membrane H⁺-ATPase and with other P-type ATPases have indicated that the isolated enzyme can function as a monomer *in vitro* (48–50). Further analysis of the mutated *pma1* genes from the *N. crassa* ccr strains may yield data that could help to resolve this controversy. The different ccr isolates have subtle differences in phenotype, suggesting that they are mutated at different sites within the protein coding region. The instability of the isolated enzyme from the ccr strains suggests that the enzyme is not folded normally and that sites that are protected in the wild-type are exposed in the mutants. It is conceivable that *in vivo* the enzyme can dynamically change between monomeric and dimeric forms. A precedent exists for such a model. The Ca²⁺-ATPase of erythrocytes (a P-type ATPase) has been reported to assume two active conformations, a dimer and a calmodulin-monomer complex (51).

CCA Inhibits the Function of the Vacuole in Vivo—Fluorescent amines such as chloroquine and quinacrine are commonly used to view acidic compartments in cells (52). Chloroquine accumulates in small vacuoles of *N. crassa* in the absence of CCA but fails to accumulate in the presence of CCA. In this paper we report another simple assay for vacuolar function. Arginine and other basic amino acids are known to accumulate to high concentrations in vacuoles of fungal cells, and this accumulation depends upon the activity of a V-ATPase in the vacuolar membrane (38, 40). We have observed that addition of CCA to the growth medium lowers the amount of arginine in the cell by 85%.

The antibiotic also had a striking effect on the morphology of cells. The hyphal branching pattern was highly disrupted. Normal tip growth in fungi involves a number of processes including cell wall digestion, cytoplasmic streaming, and organelar distribution, and depends on an organized actin cytoskeleton. Although not understood in detail, there is a body of evidence implicating cytosolic free Ca²⁺, or a Ca²⁺ gradient, as necessary for normal apical extension and branching in fungi (reviewed in Ref. 53). Raising the cytosolic...
calcium has been reported to inhibit hyphal extension and enhance branching, an effect similar to that caused by CCA. Localized pH differences have also been reported to affect tip growth (54). These observations indicate that a functional V-ATPase is necessary for normal growth patterns in N. crassa. Sequestration of Ca\(^{2+}\) in the vacuole depends on activity of the V-ATPase (55, 56), and a likely mechanism for the V-ATPase to exert its effect on tip growth is by regulating cytosolic Ca\(^{2+}\) concentrations or pH.

These data suggested that CCA was a potent inhibitor of the V-ATPase in vivo. We have recently observed in N. crassa that an inactivated uma-1 gene behaves like a simple recessive lethal mutation (57). Therefore we were surprised that cells were able to grow fairly well in the presence of CCA when tested in normal growth medium. The effect of CCA on the wild-type strain of N. crassa is reminiscent of the phenotype of uma-delete strains of S. cerevisiae, which show a pH-conditional phenotype (58). In the standard medium (pH 5.8) the addition of CCA inhibited the growth of N. crassa by approximately 50%. In alkaline medium (pH 7.2 or higher), addition of CCA stopped the germination of conidia, and no growth occurred. However, if the conidia were given time to germinate before the CCA was added, then the cells were able to grow, although at a slower rate.

If the V-ATPase is essential for growth in N. crassa, as suggested by the genetic experiments, then why are cells able to survive when the V-ATPase is inhibited by CCA? It is possible that the inhibitor is not 100% effective, but other explanations must also be considered. One plausible explanation is that the V-ATPase is required for germination of ascospores. It may not be possible to isolate strains lacking a functional V-ATPase because the spores containing the inactivated uma gene do not germinate. A different kind of explanation may also be applicable. A consequence of inactivating the uma gene is that the vacuoles will not contain any V-ATPase, which is a major protein component of the vacuolar membrane. In contrast, the entire V-ATPase complex is presumably present, although non-functional, when strains are inhibited by CCA. Although not essential in yeast, perhaps the presence of the protein itself is essential in N. crassa, either for the structural integrity of the vacuole or because the protein has another essential function. An intriguing alternative explanation was suggested by analysis of the effects of CCA on several membrane processes in Dictyostelium discoideum (16). CCA induced an alkaline shift in the pH of the endolysosomal system and inhibited endocytosis, exocytosis, and phagocytosis. The remarkable finding in these experiments, however, was that both the shift in pH and the inhibition phenomena were transient; cells recovered after 60 min. The mechanism of adaptation has not been elucidated, but a similar mechanism may be operating in the growth of N. crassa at pH 5.8 in the presence of CCA.

**Mutations in pma1 Suppress Inhibition of Growth by CCA**—Our data present the intriguing question of why mutations in the pma1 gene allow N. crassa to germinate and grow in the presence of CCA in alkaline medium. The results strongly suggest that in the mutant strains CCA still enters the cells and inactivates the V-ATPase. Although not stopped, growth is slowed by CCA, the cells do not accumulate arginine, and they do not show fluorescent vesicles in the presence of chloroquine. Furthermore, the hyphal morphology is grossly altered, just as in the wild-type.

We suggest that the altered plasma membrane H\(^+\)-ATPase is able to compensate for perturbations in cytosolic pH and calcium concentrations caused by inactivation of the V-ATPase.

**FIG. 7.** Effect of CCA on hyphal morphology at pH 7.2 after 48 h of growth. Four ccr strains (ccr40-8A, ccr6-25a, ccr1-15a, and ccr21-2a) were grown on agar plates at pH 7.2 in the presence of 0.2 \(\mu\)M CCA for 48 h to show the drastic effects of CCA on morphology. Bar, 100 \(\mu\)m.
A basic function of the fungal vacuole is to store high concentrations of ions, including Ca\(^{2+}\) and basic amino acids (38, 39). One consequence of mutation of VMA genes in yeast was that the cytoplasmic concentration of Ca\(^{2+}\) rose from 150 to 80 to 900 \(\pm 100\) nM (56). Fungal cells with inactive V-ATPases might also face with an increase in cytoplasmic pH and an increase in concentration of basic amino acids in the cytoplasm (59, 60). The plasma membrane H\(^{+}\)-ATPase might act to prevent accumulation of toxic levels of Ca\(^{2+}\), protons, or basic amino acids in the cytoplasm by driving transport systems that pump them out of the cell.

The striking difference between plasma membrane H\(^{+}\)-ATPase activities from wild-type and CCA-resistant strains was the shift from a sigmoid to a hyperbolic response to MgATP concentration. The V-ATPase of yeast is that activity of the plasma membrane H\(^{+}\)-ATPase led to a lower amount of ATPase protein and activity that cells growing in an acid medium were able to survive. Subsequently, however, Na\(^{+}\) accumulation of toxic levels of Ca\(^{2+}\), protons, or basic amino acids in the cytoplasm by driving transport systems that pump them out of the cell.

In vivo, the V-ATPase is a major ion pump in fungal cells. Be-
Concanamycin-resistant Mutants of *N. crassa*

56. Ohya, Y., Umemoto, N., Tanida, I., Ohta, A., Iida, H., and Anraku, Y. (1991) *J. Biol. Chem.* 266, 13971–13977
57. Ferea, T. L., and Bowman, B. J. (1996) *Genetics* 143, 147–154
58. Nelson, H., and Nelson, N. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3503–3507
59. Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988) *J. Bacteriol.* 170, 2683–2686
60. Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988) *J. Bacteriol.* 170, 2687–2691
61. Slayman, C. L. (1973) *J. Bacteriol.* 114, 752–766
62. Munn, A. L., and Riezman, H. (1994) *J. Cell Biol.* 127, 373–386
63. Na, S., Hincapie, M., McCusker, J. H., and Haber, J. E. (1995) *J. Biol. Chem.* 270, 6815–6823
64. Sanders, D., and Slayman, C. L. (1982) *J. Gen. Physiol.* 80, 377–402
Mutations of pma-1, the Gene Encoding the Plasma Membrane H⁺-ATPase of Neurospora crassa, Suppress Inhibition of Growth by Concanamycin A, a Specific Inhibitor of Vacuolar ATPases

Emma Jean Bowman, Forest J. O'Neill and Barry J. Bowman

J. Biol. Chem. 1997, 272:14776-14786.
doi: 10.1074/jbc.272.23.14776

Access the most updated version of this article at http://www.jbc.org/content/272/23/14776

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 44 of which can be accessed free at http://www.jbc.org/content/272/23/14776.full.html#ref-list-1