Mutations in Subunit c of the Vacuolar ATPase Confer Resistance to Bafilomycin and Identify a Conserved Antibiotic Binding Site*

Barry J. Bowman and Emma Jean Bowman
From the Department of Molecular, Cell, and Developmental Biology, University of California, Santa Cruz, California 95064

Bafilomycin A1, a potent inhibitor of vacuolar H⁺-ATPases (V-ATPase), inhibited growth of Neurospora crassa in medium adjusted to alkaline pH. Ninety-eight mutant strains were selected for growth on medium (pH 7.2) containing 0.3 or 1.0 μM bafilomycin. Three criteria suggested that 11 mutant strains were altered in the V-ATPase: 1) these strains accumulated high amounts of arginine when grown at pH 5.8 in the presence of bafilomycin, which encodes the proteolipid subunit c of the V-ATPase, and 3) V-ATPase activity in purified vacuolar membranes was resistant to bafilomycin. Sequencing of the genomic DNA encoding vma-3 identified the following mutations: T32I (two strains), F136L (two strains), Y143H (two strains), and Y143N (five strains). Characterization of V-ATPase activity in the four kinds of mutant strains showed that the enzyme was resistant to bafilomycin in vitro, with half-maximal inhibition obtained at 80–400 nM compared with 6.3 nM for the wild-type enzyme. Surprisingly, the mutant enzymes showed only weak resistance to concanamycin. Interestingly, the positions of two mutations corresponded to positions of oligomycin-resistant mutations in the c subunit of F₁F₀-ATP synthases (F-ATPases), suggesting that bafilomycin and oligomycin utilize a similar binding site and mechanism of inhibition in the related F- and V-ATPases.

The vacuolar (H⁺)-ATPase (V-ATPase) is a large, complex enzyme that couples the hydrolysis of ATP to the transport of protons across membranes. In eucaryotic cells, this enzyme plays a role in many physiological processes. It is present in several types of cellular organelles such as vacuoles, lysosomes, coated vesicles, Golgi, and secretory vesicles (reviewed in Refs. 1–3), and it is also the major proton pump in the plasma membrane of specialized acid-secreting cells such as osteoclasts and kidney intercalated cells and the intestinal epithelia cells of some insects (4). The role of the V-ATPase in physiological processes has often been examined by measuring the effects of bafilomycin and concanamycin, macrolide antibiotics that are potent inhibitors of the enzyme (5). Effective at nanomolar concentrations in vitro, these two drugs also inhibit the V-ATPase in living cells, although higher concentrations, typically 0.1–10.0 μM, are required (6). At low concentrations, the two antibiotics appear to be highly specific for V-ATPases; at 10,000-fold higher concentrations, they inhibit some P-type ATPases in vitro (7, 8).

Because of their effectiveness and specificity in vivo, bafilomycin and concanamycin are attractive candidates for development as therapeutic agents (9, 10). For example, considerable effort has been made to develop bafilomycin derivatives for the treatment of osteoporosis (11–14). Bafilomycin and concanamycin are also potent anti-tumor agents that exhibit significant cell line specificity (15). Thus, derivatives of these inhibitors might be effective in treating cancer. The pharmacological potential of these drugs has prompted several laboratories to develop methods for total synthesis of both bafilomycin and concanamycin (16–18).

The binding site of these V-ATPase inhibitors has not been determined. At least 13 different polypeptides are required for a functional enzyme. Eight types of subunits (A–H) form the V₁ sector, a ball and stalk structure that protrudes from the membrane. Five other types of subunits (a, c, c', c'', and d) form the V₀ sector, which conducts protons through the membrane. In the proposed rotary mechanism for the V-ATPase, the c', c'', and c subunits are part of a rotor, driven by the hydrolysis of ATP in the V₃ sector (19). The d and a subunits are stationary within the membrane. Proton transport is hypothesized to occur at the interface between the a subunit and the rotating c', c'', and c subunits. Several investigators have proposed that bafilomycin and concanamycin bind to the V₀ sector. Adding an excess of the V₃ sector, but not of the V₁ sector, protected the enzyme against inhibition (20, 21). Similar results were obtained using isolated subunit a, suggesting that this subunit contains a bafilomycin binding site (22). Other data indicate that the small proteolipid subunits may have a binding site. An affinity column with bafilomycin as the fixed ligand specifically bound a 17-kDa subunit (presumably subunit c) (23). Similarly, bafilomycin was reported to block proton flow through the isolated V₀, and trinitiated bafilomycin was found to copurify with the V₀ sector (20).

The inhibition of V-ATPase by bafilomycin and concanamycin in some ways resembles the inhibition of certain F₁F₀-ATP syntheses (F-ATPases) by oligomycin and venturicidin. Although their structures are significantly different (Fig. 1), all of these inhibitors are macrolide antibiotics that bind to the integral membrane sector of their respective enzymes with high specificity and with similar affinities (25, 26). F- and V-ATPases evolved from a common ancestor, raising the possibility that both types of enzymes possess an antibiotic binding site at a similar location. Mutations that confer resistance to oligomycin and venturicidin lie in the a and c subunits. The c', c'', and c subunits of V-ATPases are clearly homologs of the c subunit of F-ATPases, with a significant degree of sequence similarity (27, 28). The a subunits of the two ATPases are not similar in

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† To whom correspondence should be addressed: Dept. of MCD Biology, University of California, Santa Cruz, CA 95064. Tel.: 831-459-2245; Fax: 831-459-3139; E-mail: bowman@biology.ucsc.edu
‡ The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; P-ATPase, F₁F₀-ATP synthase; DCCD, dicyclohexylcarbodimide; bfr, bafilomycin-resistant.

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sequence but have been hypothesized to have the same structural and functional role (29).

We previously reported the isolation of mutant strains of the fungus *Neurospora crassa* that were resistant to concanamycin (6). Although we obtained resistant strains, none of them had mutations that changed the affinity of the V-ATPase for the inhibitor. The mutations allowed the organism to overcome the toxic effects of concanamycin, although the drug still entered the cells and inhibited the V-ATPase. In this report, we have used bafilomycin to screen for drug-resistant mutations. These experiments have yielded a new class of mutant strains in which the affinity of the V-ATPase for the antibiotic is altered.

**EXPERIMENTAL PROCEDURES**

*N. crassa* Strains, Growth of Cells—Strains 74A and 74a were used as the wild type. The *am al-3* strain, which is an amino acid auxotroph and an albino color mutant, was used for genetic mapping. Strains were maintained on Vogel’s medium N (a minimal medium salt solution at pH 5.8) supplemented with 2% sucrose (30). Alanine (200 mg/liter) was also added when growing strains with the *am* mutation. For experiments designed to test resistance to bafilomycin, this medium was modified by the addition of 20 mM HEPES buffer and was adjusted to pH 7.2 with KOH. Bafilomycin from a 10 mM stock in dimethyl sulfoxide was added after the medium was autoclaved. To determine the rate of growth, 4 liters of Vogel’s medium was inoculated with $10^6$ conidia (asexual spores)/ml and aerated vigorously at 25 °C. The dry weight of 50–100-ml samples was determined during the exponential phase of growth (11–18 h after inoculation). Standard methods for growth, mutagenesis, and genetic analysis of *N. crassa* were those of Davis (30).

**Mutagenesis and Selection of Mutant Strains**—Conidia from wild-type strain 74A were mutagenized by exposure to ultraviolet light and spread on agar plates containing Vogel’s medium, adjusted to pH 7.2, and 0.3 or 1.0 μM bafilomycin. After 1–3 days at 25 °C, 98 of the larger, more vigorous colonies were transferred to 2-ml agar slants of the same medium, containing 0.3 μM bafilomycin. To determine whether the resistance to bafilomycin was heritable and to generate homokaryotic strains, each of these cultures was back-crossed with the 74a strain. Progeny from each mating were then tested by germinating ascospores on agar plates containing Vogel’s medium, pH 7.2, with 0.6 μM bafilomycin. Three to five strongly growing colonies were transferred to Vogel’s medium, pH 5.8, and retested by spotting conidia on Vogel’s medium, pH 7.2, with 1.0 μM bafilomycin. Growth was scored after 2 days at 30 °C. The bafilomycin-resistant strains were mated to the *am al-3* strain to determine whether the mutation was linked to the *vma-3* locus, which encodes subunit c of the V-ATPase (31). Most progeny were scored by color and by growth on medium containing bafilomycin. For a few strains, linkage to the *am* locus was also scored by comparing growth on medium containing glycine versus medium containing ala-
nine. Representative strains of the different types of \textit{vma-3} mutations (see "Results") were back-crossed to the 74 strain a second time.

\textbf{Arginine Content of Cells—} Cultures were inoculated with 10^6 conidia/10 ml of Vogel's medium N, supplemented with 2% sucrose. Bafilomycin was added to the medium at the concentrations indicated under "Results." The cultures were shaken for 2 days at 25°C. Harvesting of the cells and determination of arginine contents were as described (6).

\textbf{Isolation of Vacuolar Membranes, Analysis of ATPase Activity, and the Effect of Inhibitors—} Vacuolar membranes were prepared as described (32) and modified (6). Protein and ATPase activities were assayed as described (6), except that assays were typically done at 37°C.

Vacuolar membranes were prepared as described (6). The protein and ATPase activities of vacuolar membranes were assayed as described (6), except that assays were typically done at 37°C. Bafilomycin and concanamycin were added to assay mixtures from 10 mM stock solutions in dimethyl sulfoxide. Dicyclohexylcarbodiimide (DCCD) was added from a 100 mM stock solution in ethanol. In all assays, the reaction was started by the addition of vacuolar membranes.

\textbf{Isolation and Sequencing of DNA—} DNA was prepared from 20 mg of lyophilized mycelia, using the DNeasy Plant Kit (Qiagen). The protein coding region of the \textit{vma-3} gene was amplified by the polymerase chain reaction. The 5′ primer was CACGGCAATCTCCAATTC, and the 3′ primer was the reverse complement of GAACCTGGTGCTAGGTCTCC. Amplified DNA of 1,080 bp was purified by separation on agarose gels and extraction by the GeneClean procedure (Bio101). The same primers were used for the sequencing reactions, performed by the DNA Sequencing Facility of the University of California, Berkeley.

\textbf{Materials—} The bafilomycin A1 used in almost all of these experiments was a gift from Dr. C. Farina and Dr. S. Gagliardi (SmithKline Beecham, Milan, Italy). In a control experiment with the bafilomycin-resistant strain bfr89, we observed the same degree of inhibition of ATPase activity with bafilomycin A1 obtained from Kamiya Biomedical Co. Bafilomycins B1, C, and D, were a gift from Dr. K. Altenhof (University of Osnabrück) and Dr. A. Zeeck (University of Göttingen). Concanamycin A1 came from Kamiya Biomedical Co.

\section*{RESULTS}

\textbf{Selection of Bafilomycin-resistant Strains—} To determine conditions for the selection of mutant strains, conidia were spread on plates containing growth medium adjusted to pH 7.2 and 0.01–3.00 \textmu M bafilomycin. At a concentration of 0.3 \textmu M bafilomycin, ~0.1% of the conidia germinated and formed tiny colonies after 2 days. Fewer than 0.01% of the conidia grew in medium with 1.0 \textmu M bafilomycin. To select mutant strains, conidia of strain 74A were exposed to ultraviolet irradiation and spread on plates containing either 0.3 or 1.0 \textmu M bafilomycin. After 2–3 days, many conidia had germinated and formed tiny colonies with highly branched hyphae, even on the higher concentration of bafilomycin. Selecting larger colonies with more normal morphology, we isolated 70 colonies in the first 2 days and an additional 28 colonies from the third day. The isolates were designated bfr1–bfr98 (for bafilomycin-resistant).

All of the isolates were mated to the wild-type strain 74a. Eighty-seven of the 98 bfr isolates produced ascospores that germinated on growth medium adjusted to pH 7.2 containing 0.6 \textmu M bafilomycin. Some of the isolates produced tiny colonies, indicating that the bafilomycin greatly restricted their growth, whereas other colonies grew more vigorously on the bafilomycin. As shown in Fig. 2, the most vigorous isolates produced colonies that were slightly larger than the best of the concanamycin-resistant mutant strains we had previously isolated (6). Isolates were selected from the germinated ascospores and tested further by spreading conidia on plates containing 1.0 \textmu M bafilomycin. Fifty-five of the strains were scored as significantly resistant to bafilomycin.

\textbf{Assessment of Bafilomycin Resistance in Vivo and in Vitro—} To determine whether the \textit{V-ATPase} was resistant to bafilomycin in vivo, we measured the concentration of arginine in bfr strains grown in liquid medium containing 0.3 \textmu M bafilomycin. In \textit{N. crassa}, large amounts of arginine are accumulated in the vacuoles by a mechanism that depends on the function of the \textit{V-ATPase} (6, 33, 34). In previous experiments in which we selected for resistance to concanamycin, none of the resistant strains had mutations in genes encoding the \textit{V-ATPase}. Instead, they had mutations that partially suppressed the toxic effects of concanamycin (6). In these ccr strains, concanamycin still entered the cell and inhibited the \textit{V-ATPase}, as evidenced by the fact that the cells had only 15–20% of the normal concentration of arginine when concanamycin was added to the medium. By contrast, in 43 of the bfr strains the concentration of arginine was at least double that of the wild type strain 74A when grown in the presence of bafilomycin. From this group of isolates we chose 22 strains with higher arginine levels for further analysis (Table I).

Other investigators have suggested that bafilomycin and concanamycin may bind to the e subunit of the \textit{V-ATPase} (23), which is encoded by \textit{vma-3} in \textit{N. crassa} (31). Because \textit{vma-3} is closely linked to the \textit{am} (amidation) and \textit{al-3} (albinogene) genes, 21 of the bfr strains described above were mated to the \textit{am} \textit{al-3} strain. In the bfr21 strain, resistance to bafilomycin mapped between the \textit{am} and \textit{al-3} loci, 2 map units (2% recombination) from each, precisely where the \textit{vma-3} gene is located. Ten other bfr strains showed tight linkage to \textit{al-3} (0–5% recombination); three bfr strains showed loose linkage (25–30% recombination); and seven bfr strains showed no linkage (~50% recombination) (Table I).

To test the effect of bafilomycin on \textit{V-ATPase} activity in \textit{vivo}, vacuolar membranes were prepared from nine of the bfr strains that showed tight linkage to \textit{al-3}, the putative \textit{vma-3} mutants, and from seven other bfr strains (Table I). In all nine putative \textit{vma-3} mutant strains, the vacuolar \textit{ATPase} was resistant to bafilomycin in \textit{vivo}. The concentration required for half-maximal inhibition was 10–20-fold higher than in the wild-type strain (data not shown). In the seven other bfr strains tested, the sensitivity of \textit{V-ATPase} activity to bafilomycin was the same as in the wild-type strain.

\textbf{Mutations in the \textit{vma-3} Gene in \textit{bfr} Strains—} To determine whether the \textit{vma-3} gene was mutated in the 11 bfr strains that showed tight linkage of resistance to \textit{al-3}, the region corresponding to the open reading frame was amplified by PCR and sequenced as described under "Experimental Procedures." In all 11 strains, a single codon in the \textit{vma-3} gene was altered (Table II). In two bfr strains, Thr^{12} was changed to Ile, and in two others Phe^{136} was changed to Leu. In seven strains, Tyr^{143} was changed either to Asn or to His. Thr^{32} is in the loop region...
interesting because they correspond to the positions of mutations that confer resistance to oligomycin and/or venturicidin in the homologous F-ATPases.

One strain was selected for each type of mutation: bfr21 (F136L), bfr33 (T32I), bfr65 (Y143H), and bfr89 (Y143N). The four were back-crossed a second time to strain 74A and used for subsequent characterization.

### Characteristics of bfr Strains with Mutations in vma-3: Effect of Bafilomycin AI

The bfr21, bfr33, bfr65, and bfr89 strains had normal hyphal morphology and fertility. Strains bfr33, bfr65, and bfr89 produced fewer conidia, in part because erratic sections of aerial mycelia failed to differentiate. Because *uma* null strains in *N. crassa* are unable to produce conidia (33), this phenotype might suggest a lower level of V-ATPase activity in the bfr mutants. The growth rate in the normal laboratory medium (Vogels, pH 5.8) was nearly the same as the wild type for bfr21, but bfr33, bfr65, and bfr89 had 15–40% longer doubling times (Table III). The effect of the mutations on vacuolar function and resistance of the V-ATPase to bafilomycin in *vivo* was examined by measuring the amount of arginine in cells after growth in varying concentrations of bafilomycin (Fig. 3).

The cells were grown in medium adjusted to pH 5.8, conditions in which the wild-type strain grows well, even in the presence of bafilomycin. The arginine concentration in the wild-type strain decreased by 50% in 0.1 μM bafilomycin and by 88% in 1.0 μM bafilomycin. By contrast, the bfr strains were able to accumulate significant levels of arginine in the presence of bafilomycin. It appeared to take 10–20-fold more bafilomycin to decrease the arginine to the levels observed in the wild-type strain. However, these data also showed that the bfr strains were partly defective in their ability to accumulate arginine. In the absence of the inhibitor, the arginine concentrations were 58–77% of the levels observed in the wild-type strain. These results suggested that the mutations in the *uma*-3 gene lowered the effective activity of the V-ATPase in *vivo*.

Vacular membranes from the four types of *uma*-3 mutant strains were assayed for V-ATPase activity and resistance to bafilomycin in *vivo*. Because the specific activity of vacuolar membranes can vary 2-fold in different preparations, it is difficult to compare rigorously the activity of different strains. In several experiments, the four bfr strains had 50–80% of the activity of the wild-type strain, and the data in Table III are typical. The effect of bafilomycin on the V-ATPase activity was significantly different in the bfr and wild-type strains (Fig. 4). bfr21, bfr65, and bfr89 showed similar levels of resistance, requiring ~13–20-fold higher concentrations of bafilomycin to inhibit 50% of V-ATPase activity. These three strains had mutations in the fourth membrane helix, near the putative proton binding residue, Glu-138. bfr33, mutated in the loop between helix 1 and 2, required ~70-fold higher concentrations of bafilomycin to inhibit 50% of V-ATPase activity. As described below, the V-ATPase activity was fully sensitive to concanamycin.

### Effect of Concanamycin, Other Forms of Bafilomycin, and DCCD on the bfr Strains with Mutations in *uma*-3

Because...
the structures of bafilomycin and concanamycin are very similar, we predicted that the V-ATPase of bfr strains would also be resistant to concanamycin A. To our surprise, the V-ATPase in bfr strains was not greatly altered in sensitivity to concanamycin. All of the strains were assayed at the same time in the same batch of solutions (Fig. 5). The bfr21 strain showed essentially the same sensitivity to concanamycin A as the wild-type strain. bfr33, bfr65, and bfr89 were slightly resistant, requiring 3-fold higher concentrations of concanamycin to inhibit 50% of V-ATPase activity.

The major structural difference between bafilomycin and concanamycin is in the macrolide ring. If the bfr strains discriminated between these two classes of antibiotics, then we predicted that V-ATPase of the bfr strains should be resistant to other forms of bafilomycin. We tested the effects of bafilomycin B1, C, and D, as shown in Table IV. The bfr strains were
Mutations in Subunit c Confer Resistance to Bafilomycin

**Table IV**

| V-ATPase activity | 74A | bfr21 | bfr33 | bfr65 | bfr89 |
|-------------------|-----|-------|-------|-------|-------|
| %                 |     | 100   | 100   | 100   | 100   |
| 10 nM Baf A1      | 100 | 100   | 98    | 90    | 85    |
| 100 nM Baf A1     | 4   | 46    | 79    | 55    | 50    |
| 10 nM Baf B1      | 100 | 100   | 100   | 100   | 94    |
| 100 nM Baf B1     | 28  | 62    | 81    | 79    | 70    |
| 10 nM Baf C       | 100 | 100   | 101   | 101   | 101   |
| 100 nM Baf C      | 50  | 83    | 92    | 91    | 85    |
| 10 nM Baf D       | 96  | 98    | 101   | 101   | 95    |
| 100 nM Baf D      | 84  | 96    | 100   | 100   | 97    |
| 1000 nM Baf D     | 35  | 85    | 98    | 86    | 83    |

significantly resistant. The relative potency of the different bafilomycins was consistent with previous results (7, 8, 35).

In experiments with F-ATPases, it has been shown that some mutations in the c subunit that confer resistance to oligomycin also increase the affinity of the enzyme for DCCD (reviewed in Ref. 25). We examined the effects of DCCD on V-ATPase activity in the vacuolar membranes (Fig. 6). The four bfr strains were partially resistant to the toxic effects of bafilomycin. In the fourth box, the glutamate residue that has been proposed as a site of proton binding is underlined (27). Only the region around the third membrane helix of subunit c is shown because there is not sufficient sequence similarity to align unambiguously the other parts of subunit c with subunits c and c'

**Figure 6.** The V-ATPase of bfr strains is unchanged in its sensitivity to DCCD. The effect of DCCD on V-ATPase activity in purified vacuolar membranes (2 μg protein) was assayed at 37 °C. The wild type (74A) and four bfr strains were tested at the same time with the same assay mix. Specific activities in the absence of inhibitor were 2.7 μmol/min/mg (74A), 2.1 μmol/min/mg (bfr21), 2.5 μmol/min/mg (bfr33), 2.8 μmol/min/mg (bfr65), and 3.7 μmol/min/mg (bfr89). Half-maximal inhibition by DCCD was achieved at 13 μM for 74A, 15 μM for bfr21, 21 μM for bfr33, 13 μM for bfr65, and 13 μM for bfr89.

**Figure 7.** Alignment of protein sequences of subunits c, c', and c" from *N. crassa*. The sequences of the c', c", and c" subunits of *N. crassa* were derived from DNA sequences in the data base of the Neurospora Genome project at the Whitehead Institute for Biomedical Research. Boxed regions represent putative membrane-spanning domains. The boxes mark the residues that are mutated in the bfr strains. In the fourth box, the glutamate residue that has been proposed as a site of proton binding is underlined (27). Only the region around the third membrane helix of subunit c" is shown because there is not sufficient sequence similarity to align unambiguously the other parts of subunit c" with subunits c and c'.

**Discussion**

By selecting for growth on alkaline medium containing bafilomycin, we obtained two types of mutant strains that were partially resistant to the toxic effects of bafilomycin. In most of the mutant strains, the V-ATPase did not appear to be altered. Bafilomycin inhibited the enzyme in vitro and lowered the accumulation of arginine in the vacuoles in vivo. In a subset of these strains, the level of arginine in cells exposed to bafilomycin was higher than in the wild-type strain treated the same way. These strains may be able to exclude or inactivate the bafilomycin. We are currently mapping the location of the mutations that confer resistance without altering the V-ATPase and plan to characterize them further.

Several laboratories have reported that bafilomycin binds to the V0 sector of the V-ATPase. Rautiala et al. (23) showed that an affinity column with bafilomycin as the fixed ligand specifically bound a 17-kDa proteolipid subunit. In our experiments, in the 11 mutant strains that showed the highest degree of resistance (e.g. formed the largest colonies on medium with bafilomycin), mutations were found in the vma-3 gene, encoding the c subunit of the V-ATPase. The isolated enzymes showed a 13–70-fold increase in the $K_i$ for bafilomycin. In the vma-3 mutant strains, at least 20-fold higher levels of bafilomycin were required to inhibit the accumulation of arginine in vivo. These data strongly suggest that the c subunit is a part of the bafilomycin binding site. Furthermore, the finding that these single mutations confer resistance in vitro indicates that bafilomycin is highly specific for the V-ATPase in vivo. The many effects it has on physiological processes in the cell are likely to be secondary consequences of the inhibition of the V-ATPase.

Given the high degree of similarity in the structures of bafilomycin and concanamycin (Fig. 1), we were surprised that mutations that conferred bafilomycin resistance had much smaller effects on the affinity of the V-ATPase for concanamycin. In some organisms, such as the plant *Mesembryanthemum crystallinum*, bafilomycin and concanamycin inhibit with very similar potencies, whereas in other organisms such as *N. crassa* concanamycin is 4–10-fold more potent (7, 35). We had assumed that these two drugs bound to the same site, but the V-ATPase from the bfr strains clearly discriminated between them. These two antibiotics do have significant differences in...
structure that could explain binding to different sites or at least different affinities for a particular site. For example, the macro lactone ring in concanamycin is 2 carbons larger than that in bafilomycin. The dienic systems in the macrolactone ring have been shown to be essential for inhibitory activity (35, 36).

One interesting possibility is that bafilomycin and concanamycin may bind to subunits c, c/H11032, and/or c/H11033 but with different affinities. These three polypeptides have similar amino acid sequences, especially in the region in which bfr21, bfr65, and bfr89 are mutated (Fig. 7). Subunits c/H11032 and c/H11033 also have Phe and Tyr residues in positions corresponding to Phe136 and Tyr143 in subunit c. Thr32 of subunit c, mutated in bfr33, aligns with a Thr in subunit c/H11032. When comparing the first three transmembrane helices of subunits c and c/H11032 with subunit c/H11033, there is not sufficient sequence similarity to allow an unambiguous alignment. However, none of the possible alignments suggest that subunit c/H11032 has a conserved Thr in an equivalent position to Thr32 of subunit c. Antibiotic binding to multiple c subunits could explain why all of the bfr strains retain a significant sensitivity to bafilomycin. If concanamycin binds to c and c/H11032 with nearly equal affinities, it may be more difficult to obtain mutants resistant to concanamycin, because at least two genes would have to be altered at specific sites. We are currently testing these ideas by screening for additional mutants and by site-directed mutagenesis of the c/H11032 and c/H11033 subunits.

Although the structure of subunit c in the V-ATPase has not been experimentally determined, it is clearly a homolog of subunit c in the F-ATPase (28). The predicted structures consist of two or four α-helices that cross the lipid bilayer, connected by short hydrophilic loops (Fig. 8). The glutamic acid residue in the middle of helix 2 (F-ATPase) or helix 4 (V-ATPase) is postulated to play a key role in the transport of protons (27, 37). Two of the residues altered in the bfr mutant strains lie close to Glu-138. Mutation of Phe136 to Leu or of Tyr143 to Asn or His conferred resistance to bafilomycin. If this region of the polypeptide is modeled as an α-helix (Fig. 9), the mutated residues appear to be on the same face of the helix, 11 Å apart, and on the opposite side from Glu138. Conceivably, the aromatic rings in Phe136 and Tyr143 are important in binding the macrolactone ring of bafilomycin. The position of Thr32 in the tertiary structure is difficult to predict, but the polypeptide could be folded in a way to place this residue near the fourth helix.

A large body of work from the 1970s and 1980s identified residues involved in oligomycin binding in the c subunit of mitochondrial ATPases from Saccharomyces cerevisiae and N. crassa. A large body of work from the 1970s and 1980s identified residues involved in oligomycin binding in the c subunit of mitochondrial ATPases from Saccharomyces cerevisiae and N. crassa.
Mutations in subunit c confer resistance to bafilomycin

FIG. 9. Helical wheel model of a region of the fourth membrane helix in subunit c. The model shows the location of the side chains of residues 133–150. Glu^{138} is the putative proton-binding site. In bfr strains residue Phe^{136} was mutated to leucine, and Tyr^{143} was mutated to either asparagine or histidine.

crassa (25, 26). There is a striking correspondence between the positions of mutated residues in the bfr strains and the positions of mutations that confer resistance to oligomycin in F-ATPase (Fig. 8). Using the putative proton-binding glutamate as a point of alignment, Phe^{136} and Tyr^{143} are in precisely the same positions as Leu^{37} and Phe^{64} of the F-ATPase in S. cerevisiae. Likewise, a mutation in the loop region of the F-ATPase c subunit also conferred resistance to oligomycin. This raises the interesting possibility that F-ATPase and V-ATPase share a critical interface with subunit a. High affinity binding of anti-biotics could prevent the proposed conformational changes within subunit c or the rotation of c relative to subunit a. The evolution of this complex rotary mechanism may have been accompanied by the evolution of a variety of antibiotics that take advantage of a vulnerable site within the F-ATPases and V-ATPases.

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3972
