Decreased Activity of UMP Pyrophosphorylase Associated with Resistance to 5-Fluorocytosine in *Candida albicans*

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UMP pyrophosphorylase activity was assayed in crude lysates prepared from *Candida albicans*. Specific activity of UMP pyrophosphorylase was high in clinical isolates which were susceptible to 5-fluorocytosine. Resistant clinical isolates displayed low activity, and partially resistant (heterozygous) isolates displayed intermediate activity. Segregation from the heterozygous state resulted in a homozygous susceptible segregant with high UMP pyrophosphorylase activity and a homozygous resistant segregant with low activity. The observed specific activities were consistent with the hypothesis that specific activity was determined by the sum of the activities due to the dominant (FCY) and recessive (fcy) alleles of the resistance gene. Strains which possessed little UMP pyrophosphorylase activity released uracil into the medium when grown in the absence of 5-fluorocytosine; this result suggested that recycling of intracellular uracil is a normal function of this enzyme.

The clinical usefulness of 5-fluorocytosine (5-FC) is limited by the occurrence of resistant *Candida albicans* strains. In principle, at least three mechanisms of resistance are predictable from the known pathway of 5-FC metabolism (Fig. 1). A defect in the cytosine permease, in the cytosine deaminase, or in the UMP pyrophosphorylase, is expected to confer resistance by blocking formation of the key intermediate 5-fluoro-uridine monophosphate (FUMP). Resistance to 5-FC has not been shown in detail at the enzyme level in *C. albicans*, but instead the likely basis of resistance was inferred from patterns of resistance to fluorinated pyrimidines (5-FC, 5-fluorouracil, 5-fluorouridine). That approach (7), which used gene-enzyme studies in *Saccharomyces cerevisiae* as a reference (3), suggested that resistance to 5-FC in *C. albicans* was due in the majority of cases either to a defect in the UMP pyrophosphorylase or to a defect in regulation of de novo pyrimidine synthesis which would result in overproduction of endogenous pyrimidine nucleotides and competition with the fluorinated pyrimidine nucleotides. However, no distinction was made between these alternatives, and the enzymatic basis of resistance in resistant clinical isolates remained unknown. In another study (6), a 5-FC-resistant variant isolated in the laboratory was shown to have decreased UMP pyrophosphorylase activity by direct assay. Resistance due to a defect in the cytosine deaminase was demonstrated by assay in two isolates (7). Resistance due to a defect in 5-FC uptake has not been reported in *C. albicans*, but weak resistance due to an uptake defect has been shown in *S. cerevisiae* (3) and in *Torulopsis glabrata* (D. Kerridge and W. L. Whelan, unpublished data).

The existence of distinct classes of *C. albicans* isolates in terms of 5-FC resistance was reported (1, 12); the classes were distinguished by their resistance to 5-FC at a single concentration (50 μg/ml). Type E strains (resistant) were capable of rapid growth at that concentration, whereas growth of type D strains (susceptible) was abolished. Growth of type C strains (partially resistant) was markedly but incompletely inhibited under those conditions. Resistant isolates were infrequent (6%) in a clinical survey (1), whereas partially resistant isolates occurred at significant frequency (37%), and susceptible isolates were in the majority (57%). Stiller et al. (10) also surveyed 5-FC resistance, by using different methods and isolates; they also reported the infrequent occurrence of highly resistant isolates, a significant frequency of partially resistant isolates, and a majority of susceptible isolates. Genetic analysis (12) showed that three typical partially resistant isolates were heterozygous for resistance and gave rise to mitotic segregation (FCY/fcy → FCY/FCY + fcy/fcy) to a susceptible segregant and a resistant cosegregant. In a suitably marked strain, it was shown that mitotic recombination (reciprocal) accounted for the majority of resistant segregants, and that resistant variants might also arise by gene conversion (W. L. Whelan and D. Markie, submitted for publication).

We report here that UMP pyrophosphorylase activity occurred at high levels in 5-FC-susceptible isolates, at intermediate levels in partially resistant (heterozygous) isolates, and at low levels in resistant isolates. Each heterozygote (of seven examined) gave rise to a resistant segregant which displayed decreased UMP pyrophosphorylase activity and to a susceptible cosegregant which displayed increased activity. These results indicated that clinical resistance to 5-FC is likely to be due to decreased UMP pyrophosphorylase activity in the majority of cases, and they support the hypothesis that resistant clinical strains may arise from heterozygotes.

**MATERIALS AND METHODS**

Clinical isolates. The sources of clinical isolates appear in Table 1. Classification of isolates on the basis of resistance was done as described previously (1); classification of the strains used in the present study appears in Table 1.

Media. The defined minimal medium was MIN agar (2). MFC50 agar was MIN agar with the addition of 5-FC (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 50 μg/ml; filter-sterilized 5-FC was added to autoclaved MIN agar.

Genetic methods. Sected (susceptible:resistant) colonies were obtained by irradiating cells spread on MIN agar and...
FIG. 1. Metabolism of 5-FC (reviewed in reference 9).

then replica-plating the colonies which appeared (after incubation for 2 days at 37°C) to MFC50 agar. This technique was essentially as described previously (12), except that the UV dose was 240 ergs/mm² (determined with a model J225 ultraviolet meter; Ultra-Violet Products, Inc., San Gabriel, Calif.).

UMP pyrophosphorylase activity. Cultures were grown in liquid medium (yeast nitrogen base [Difco Laboratories, Detroit, Mich.] plus 2% glucose) (100 ml) at 37°C to early stationary phase with shaking. Cells (1.3 to 1.7 g [wet weight]) were collected by centrifugation and washed once in cold water (4 ml) and once in buffer B (4 ml). Buffer B (pH 7.5) contained 0.05 M Tris-hydrochloride, 10⁻³ M EDTA, and 10⁻³ M mercaptoethanol. The pellet was suspended in buffer B (0.75 ml), and cooled, acid-washed glass beads (0.5 mm) were added to a level slightly below the meniscus. The mixture was agitated with a vortex mixer five times for 30 s each, alternated with cooling periods on ice. Microscopic examination confirmed that more than 90% of cells were broken. Liquid was aspirated from the mixture, and the beads were washed twice with 1 ml portions of buffer B; the crude lysate consisted of the aspirated liquid pooled with the washes.

UMP pyrophosphorylase (UMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.9) activity of lysates was assayed by the method of Molloy and Finch (5), except that lysates were not preincubated with GTP. Those authors found that dialyzed extracts of Escherichia coli required preincubation with GTP for expression of maximum UMP pyrophosphorylase activity, but no stimulatory effect of GTP was observed in our preliminary experiments. Reaction mixtures contained 20 µl of crude lysate plus 10 µl of uracil solution (1.6 × 10⁻³ M uracil in 10⁻³ M MgCl₂) and 10 µl of 5-phosphoribosyl-1-pyrophosphate (PRPP; Sigma) solution (1.6 × 10⁻³ M PRPP in buffer B). [2-¹⁴C]Uracil was obtained from Amersham International; the specific activity of uracil in the reaction was 13.6 Ci/mol. The reaction was started by addition of lysate to prewarmed substrate mixture and was stopped by heating (100°C, 3 min). The cooled reaction mixture was centrifuged (3 min) in a Beckman microfuge, a sample (5 µl) of supernatant was chromatographed on polyethyleneimine thin-layer sheets (Macherey-Nagel CEL300 polyethyleneimine) to separate product UMP (origin) from substrate uracil (front) by the method of Molloy and Finch (5), and radioactivity in the substrate and product was determined (see below). The activity was heat labile (100°C, 3 min), and no UMP was formed in the absence of added PRPP. Protein was determined by a modification (4) of the Lowry method, with bovine serum albumin as standard. The average deviation of UMP pyrophosphorylase specific activity (nanomoles per minute per milligram) was ±18% of the mean value for a strain (17 strains, 53 determinations).

Cytosine permease activity. Cultures of C. albicans growing aerobically at 37°C in Difco yeast nitrogen base–1% glucose were diluted into fresh, prewarmed growth medium to a density of 0.1 mg (dry weight)/ml and incubated aerobically at 37°C. After 2 h of incubation, 10-ml portions of the exponentially growing culture were transferred to flasks containing 5F¹⁴C]cytosine (specific activity, 1 Ci/mmol; concentration as required) and the incubation was continued. Samples (1 ml) were withdrawn at intervals (1, 2, 5, and 10 min) and the organisms were harvested by filtration through Whatman GF/C filters. The filters were washed twice with 5 ml of cold distilled water and dried at 105°C; the radioactivity was assayed by scintillation spectrometry with 2,5-diphenyloxazole–toluene (0.4% [wt/vol]) as scintillant and a Packard Scintillation Spectrometer model 3385.

Detection and identification of pyrimidines released into growth media. Cultures were screened for pyrimidine release by bioassay. Cells (ca. 10⁶) of a pyrimidine auxotroph (strain M4) derived from S. cerevisiae S288C were spread on MIN agar, and the C. albicans strains were inoculated in patches (ca. 1 by 1 cm); cultures were incubated at 37°C. Growth of the indicator strain in a zone around the confluent growth of a C. albicans strain was taken as evidence of pyrimidine release. Strain M4 was shown to grow in response to added uracil, uridine, or cytosine but did not grow in response to other pyrimidines or related compounds (cytidine, thymine, thymidin, orotate, dihydroorotate, dihydrouracil).

Chromatographic identification of released pyrimidines was performed in parallel on a resistant segregant (72R) and its susceptible cosegregant (72S) derived from heterozygote MEN. Cultures were grown at 37°C for 50 h in liquid medium (500 ml of vitamin-free yeast base [Difco] containing biotin [2 µg/liter] and glucose [20 g/liter]) with shaking. The cultures were centrifuged and the pellet (4.2 to 4.3 g [wet weight]) was discarded. The supernatant fluid was filtered (Whatman GF/C), a portion (100 ml) of the filtrate was adjusted to pH 10 with NH₄OH (35%) and left at room temperature for 20 min, during which time a precipitate appeared. The precipitate was removed by filtration (Whatman GF/C), the filtrate was applied to a Dowex 1X2-200 column (bed volume, 16 ml; OH form), and the column was washed with 0.01 M NH₄OH. In control experiments, standard cytosine was eluted by the 0.01 M NH₄OH wash. The column was then washed with 0.1 M acetic acid, and UV-absorbing material, which was eluted in a sharp peak corresponding in eluant volume to the peak for uracil and uridine standards, was collected and evaporated to dryness. The dried material was dissolved in water and samples were chromatographed on thin layer sheets (Macherey-Nagel CEL300UV), with n-butanol-water (86:14) as solvent. Other samples were subjected to a colorimetric determination of uracil (8).

RESULTS

UMP pyrophosphorylase activity in clinical isolates. The PRPP-dependent formation of UMP from uracil was assayed

TABLE 1. C. albicans isolates

| Resistance class | Isolates* |
|------------------|----------|
| D (susceptible)  | AD1, AD5, AD8, AD9, AD10, AD27, AD29, AD32, QC3, QC4 |
| C (partially)    | AD11, AD13, AD17, AD18, AD21, QC20, MEN |
| E (resistant)    | 407, 6844, 7963, 8016, 8233 |

* Isolates designated AD were obtained from New Addenbrooke's Hospital, Cambridge; those designated QC were obtained from Queen Charlotte's Maternity Hospital, London. The Class E isolates were obtained in a survey of resistance in the United States (1). Isolate MEN was described previously (12).
The standard reaction protocol is described and of three resistance classes: the specific activity of UMP pyrophosphorylase was high in susceptible isolates, intermediate in partially resistant isolates, and low in resistant isolates (Fig. 4).

The 5-FC-susceptible isolates studied here were a random sample of such isolates obtained in Britain and were phenotypically uniform in failing to grow discernibly in the standard screening procedure (11) on MFC50 agar; all susceptible isolates displayed high UMP pyrophosphorylase activity. The partially resistant isolates studied were typical representatives of that class; all possessed less UMP pyrophosphorylase activity than did any susceptible isolate, and all were shown (below) to be heterozygous for resistance. The resistant isolates were obtained in a previous survey of 5-FC resistance (1); the observed low UMP pyrophosphorylase activity was sufficient to account for resistance.

Segregation of resistant variants. The finding (above) that partially resistant isolates possessed less UMP pyrophosphorylase than did type D isolates was consistent with heterozygosity (FCY/fcy), on the assumption that the specific activity was determined by the sum of the activities determined by the FCY and fcy alleles present in the cell. It was expected that segregation in a typical type C isolate (FCY/fcy → FCY/FCY + fcy/fcy) would yield a susceptible segregant with the increased specific activity typical of susceptible clinical isolates and a resistant cosegregant with the decreased specific activity typical of resistant clinical isolates.

To test that hypothesis, we obtained sectored colonies (FCY/FCY; fcy/fcy) which were composed of the homozgyous products of segregation (see above); that result confirmed heterozygosity (FCY/fcy) in each partially resistant isolate. For each segregation, the susceptible (FCY/FCY) segregant displayed an increased UMP pyrophosphorylase specific activity, whereas the resistant (fcy/fcy) segregant displayed a decreased specific activity. It was evident (Table

FIG. 2. Progress curves for the formation of UMP from uracil and PRPP catalyzed by lysates prepared from a type D isolate, AD10 (O); a type C isolate, AD18 (O); and a type E isolate, 407 (Δ). The standard reaction protocol is described in the text.

in crude cell lysates prepared from early stationary phase cultures of representative clinical isolates chosen from each of three resistance classes (see above). Typical progress curves (Fig. 2) indicated that activity was readily detected in a susceptible (type D) isolate and in a partially resistant (type C) isolate, whereas little activity was evident in a resistant (type E) isolate. The reaction rate was approximately linearly related to the volume of added lysate (Fig. 3). UMP pyrophosphorylase activity was determined in lysates prepared from clinical representatives of the three resistance classes; the specific activity of UMP pyrophosphorylase was high in susceptible isolates, intermediate in partially resistant isolates, and low in resistant isolates (Fig. 4).

FIG. 3. Initial reaction rate of the UMP pyrophosphorylase activity of isolate MEN as a function of volume of lysate added to the standard substrate mixture (16 nmol of uracil plus 16 nmol of PRPP). The final volume (40 µl) was made up with lysate or with buffer B (see the text).
TABLE 2. Specific activity of UMP pyrophosphorylase in crude lysates prepared from heterozygotes (type C) and derived segregants

| Parent isolate | UMP pyrophosphorylase sp act (nmol/min per mg) |
|----------------|-----------------------------------------------|
|                | Segregants |          |          |          | Parent          |
|                | Susceptible | Resistant | Found | Expected* |
| AD11           | 2.0        | 0.1       | 1.1    | 1.0       |
| AD13           | 2.9        | 0.2       | 1.9    | 1.6       |
| AD17           | 1.8        | 0.2       | 1.3    | 1.0       |
| AD18           | 3.0        | 0.1       | 1.4    | 1.5       |
| AD21           | 3.3        | 0.2       | 1.3    | 1.8       |
| QC20           | 3.0        | 0.1       | 1.6    | 1.6       |
| MENb           | 2.8        | 0.4       | 1.3    | 1.6       |
| MEN            | 2.8        | 0.2       | 1.3    | 1.5       |

* Expected specific activity was calculated as (T + t)/2, where T is the specific activity shown by the susceptible segregant, and t is the specific activity shown by the resistant segregant.

The results obtained from two independent segregant colonies derived from isolate MEN are shown.

The resistant segregants obtained from each of six heterozygotes (isolates AD11, AD13, AD17, QC20, and MEN) were found to release pyrimidines into the growth medium; pyrimidine release was not tested in resistant segregants from heterozygotes AD18 and AD21. Twenty independent resistant variants were isolated from heterozygote MEN by selection on MFC50 agar, and all were found to release pyrimidine(s). The foregoing results indicated that pyrimidine release was associated with resistance, which was presumably due to decreased UMP pyrophosphorylase activity.

Chromatographic identification of uracil released by resistant segregant 72R (from MEN \( \rightarrow 72(S:R) \)) was performed as described above, and the same procedure was applied in parallel to the susceptible cosegregant (72S). Material which displayed the chromatographic behavior of standard uracil was detected in the culture medium of segregant 72R but was not detected in the culture medium of segregant 72S. Whether uridine was released was not clear because of poor chromatographic resolution of standard uridine from interfering materials present in both culture media. By using a colorimetric method (8), we determined that the culture medium of segregant 72R contained uracil (0.3 \( \mu g/ml \)) and uracil was not reliably detected (<0.05 \( \mu g/ml \)) in the culture medium of segregant 72S. No chromatographic test of cytosine release was made.

Cytosine permease activity. Initially the uptake of 5-Fl[14C]cytosine was studied in the heterozygous strain MEN, in resistant segregant 72R, and in susceptible segregant 72S. Transport of the drug into the organism was active and conformed to Michaelis-Menten kinetics with a \( K_m \) value for the three strains of 7 \( \mu M \) and a \( V_{max} \) of 1.5 nmol/mg (dry weight) per min. The uptake of 5-Fl[14C]cytosine into the susceptible strain AD1 and the clinically resistant strains 407, 6844, 7963, 8016, and 8223 was also studied, and similar results were obtained: none of these strains was deficient in 5-FC uptake. In agreement with a study by other workers (7), our results suggest that it is unlikely that defective 5-FC uptake accounts for a significant number of resistant strains.

**DISCUSSION**

We have shown in the present paper that 5-FC-susceptible clinical isolates possessed high levels of UMP pyrophosphorylase activity, whereas 5-FC-resistant isolates possessed little activity. Isolates which were partially resistant possessed an intermediate level of activity. The isolates studied were typical representatives of the three resistance classes described previously (1, 12). We consider, then, that the decreased UMP pyrophosphorylase activity observed is sufficient in principle to account for resistance in the majority of resistant and partially resistant clinical isolates.

Three partially resistant isolates were shown previously to be heterozygous for resistance (11), and we demonstrated heterozygosity in six additional partially resistant isolates in the present study. Our finding that those heterozygotes gave rise to resistant segregants which resembled resistant clinical isolates in possessing little UMP pyrophosphorylase activity supported the hypothesis (11) that resistant strains may be derived from partially resistant heterozygotes in clinical practice.

We have shown that each partially resistant heterozygote studied gave rise to a resistant segregant with decreased UMP pyrophosphorylase activity and to a susceptible co-segregant with increased activity. The data were consistent with the hypothesis that specific activity in a cell was determined by the sum of the activities determined by the dominant allele(s) \( FCY \) and the recessive allele(s) \( fcy \) present. Those results provided strong support for the hypothesis that hereditary variants arise by segregation from the heterozygous state, which is the basis of our approach to \( C. albicans \) genetics (reviewed in reference 11), and suggest that 5-FC resistance–UMP pyrophosphorylase will be useful in gene-enzyme studies in \( C. albicans \).

UMP pyrophosphorylase has not been well characterized in any species, and its role in metabolism has not been defined. We have found that strains which possessed little UMP pyrophosphorylase activity released uracil into the medium during normal growth in the absence of 5-FC; a similar effect was observed by Jund and Lacroute in \( S. cerevisiae \) (3). It therefore seems likely that in addition to its possible role in utilization of uracil derived from the medium, UMP pyrophosphorylase acts to recycle uracil that is formed due to catabolism of UMP by other enzymes. Activities other than UMP pyrophosphorylase which catalyze breakdown of UMP to uracil in \( S. cerevisiae \) (3). However, UMP pyrophosphorylase does not appear to be important in determining the growth rate of ordinary prototrophic strains on laboratory media; we observed no obvious variation in growth rate associated with genetically determined differences in UMP pyrophosphorylase activity. The release of small amounts of uracil by resistant strains does not appear to constitute a significant metabolic inefficiency which might result in selection against resistance.

The specific activities of UMP pyrophosphorylase determined for susceptible and partially resistant isolates in the present study were significantly higher than values obtained by other workers in \( C. albicans \) (6) and \( S. cerevisiae \) (3). A thorough characterization of UMP pyrophosphorylase seems useful, in view of its role in resistance to 5-FC.
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