Bioenergetics (1973) **4**, 161–177

Biogenesis of Mitochondria
23. The Biochemical and Genetic Characteristics of Two Different Oligomycin Resistant Mutants of *Saccharomyces Cerevisiae* Under the Influence of Cytoplasmic Genetic Modification

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Abstract

Two classes of *Saccharomyces cerevisiae* mutants resistant to oligomycin, an inhibitor of mitochondrial membrane bound ATPase are described. Biochemical analysis shows that *in vitro* the mitochondrial ATPase of both types of mutant are sensitive to oligomycin. *In vivo* sensitivity of the mutants to oligomycin can be demonstrated following anaerobic growth of the cells, which grossly alters the mitochondrial membrane and renders the ATPase of the mutants sensitive to oligomycin. It is concluded that the mutation to oligomycin resistance in both mutant types is phenotypically expressed as a change in the mitochondrial membrane. The intact mitochondrial membrane in the wild type cell is freely permeable to oligomycin, whereas the resistant mutant is impermeable to oligomycin; alteration of the mitochondrial membrane during isolation of the organelle or physiological modification of the membranes of the mitochondria by anaerobic growth renders the membranes permeable.

These mitochondrial membrane mutants differ in their cross-reference patterns and their genetics. One is resistant to oligomycin only, and behaves like previously reported cytoplasmic mutants. The other shows cross-resistance to inhibitors of mitochondrial protein synthesis as well as to oligomycin; although the mutant appears to arise from a single step mutation its genetic properties are complex and show part-nuclear and part-cytoplasmic characteristics. The implications of the observations are discussed.

Introduction

As an integral part of the study of the biogenesis of mitochondria in the yeast *Saccharomyces cerevisiae*, this laboratory has been studying the formation and function of mitochondrial membranes in yeast. Previous communications have described the manipulation of the composition of mitochondrial membranes by physiological and genetic methods and

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the studies illustrate and emphasize the critical nature of the organization of the mitochondrial membrane in the regulation of diverse mitochondrial processes (for review[1,2]).

When grown under anaerobic conditions, *S. cerevisiae* cannot synthesize unsaturated fatty acids (UFA) or ergosterol[3,4] and such cells contain poorly developed mitochondria.[5–9] They can, however, incorporate into their membranes a wide variety of unsaturated fatty acids[10] and sterols[11] enabling the lipid composition of mitochondrial and other cell membranes to be varied by altering the lipid supplements in the growth medium.[12] The normal unsaturated fatty acid content (UFA) of yeast mitochondria is about 75% of the total organelle fatty acids; cells grown to contain less than 20% UFA contain mitochondria deficient in ribosomes[13] and the DNA dependent RNA polymerase.[14] This process is freely reversible and on restoration of the mitochondrial UFA content, the mitochondrial RNA polymerase and ribosome content returns to normal.[15]

The employment of anaerobically grown cells is of limited use in the study of the energy-linked functions of mitochondria, as the anaerobic cell lacks the normal respiratory cytochromes.[16] The biochemical analysis of genetic mutants with altered mitochondrial membranes enables investigations to be carried out on the role of the membrane in the functioning of the organelles under aerobic conditions. Using a fatty acid desaturase mutant, we have shown that under aerobic conditions limited UFA-depletion (about 30% of total fatty acids) leads to a specific loss of the ability of the mitochondria to couple oxidation to the formation of ATP and that this process is reversible on the addition of UFA.[17–19] Another class of mutant which is providing some insight into mitochondrial membrane function consists of mutants with cytoplasmically determined antibiotic resistance. Recently our laboratory has reported the isolation of cytoplasmic mutants resistant to a number of antibiotic inhibitors of mitochondrial protein synthesis, namely, mikamycin, chloramphenicol, carbomycin, lincomycin and tetracycline, and these mutants have been phenotypically characterized as probable mitochondrial membrane mutants.[1,20] The investigations have led to the suggestion that extensive mitochondrial ribosome-membrane interactions occur;[1,20] the lipid depletion studies in general support this concept.[13–15]

Oligomycin resistant mutants of yeast which show cytoplasmic inheritance have been described by Wakabayashi and Gunge,[21] Stuart,[22] and Avner and Griffiths.[23] These three groups of workers report that the ATPase of mitochondria isolated from their mutants is completely sensitive to oligomycin, although more recently Griffiths *et al.*[24] have also reported that the mitochondrial ATPase from some of their mutants shows partial *in vitro* resistance to oligomycin.

This communication is concerned with the biochemical and genetic

characterization of a number of oligomycin resistant mutants. On the basis of their genetic behaviour, they fall into two classes, one classically cytoplasmic and the other a new type showing nucleo-cytoplasmic genetic interaction; they are also distinguished by their different cross-resistance patterns to a variety of chemically unrelated mitochondrial protein synthesis antibiotic inhibitors. Biochemical analysis of both genetic classes of mutant indicates that the *in vitro* and *in vivo* properties of the mitochondrial oligomycin sensitivity conferring protein (OSCP) and ATPase are unaltered, and that the mutation to oligomycin resistance is a consequence of a change in some other mitochondrial membrane component.

Materials and Methods

Isolation of Mutants

Strains described as sensitive to an antibiotic are unable to grow on a non-fermentable substrate, such as ethanol, in the presence of that drug; in particular, at concentrations per milliliter of medium, of 0·3 μg oligomycin, 0·01 mg mikamycin, 0·5 mg chloramphenicol, 0·1 mg erythromycin, 0·5 mg lincomycin, 0·3 mg carbomycin, 1·0 mg spiramycin, 0·5 mg tetracycline. Conversely, strains resistant to these antibiotics are able to grow on such media in the presence, per milliliter of medium, of 50 μg oligomycin, 0·3 mg mikamycin, 4 mg chloramphenicol, 4 mg erythromycin, 4 mg lincomycin, 0·6 mg carbomycin, 4 mg spiramycin, 2 mg tetracycline.

Cultures of the sensitive, prototrophic diploid strain, N 1300 were irradiated with ultraviolet light to give approximately a 99% kill, and the surviving cells were grown for 18 h on a yeast extract-peptone-ethanol (YEPE) medium to allow for phenotypic lag. The culture was then plated on YEPE medium supplemented with 50 μg/ml oligomycin. Resistant mutants appeared on these plates as papillae; one such diploid mutant, N 1311, was then isolated and sporulated to obtain the resistant haploid L 4000. This technique was similar to that used for the isolation from N 1300 of the mikamycin resistant mutant N 1301.[20] This mikamycin resistant diploid mutant, N 1301, and all spores from it including L 3000 examined here, were cross-resistant to oligomycin. All the mutant strains isolated were stable with respect to their antibiotic resistance.

Mating procedures, the method of tetrad analysis, and the determination of the proportion of sensitive and resistant cells that arise from single diploid zygotes (denoted by us as mixedness) have been described previously, as have the complex and synthetic media used.[25–27]

The auxotrophic haploids used in the present study are characterized by the following growth requirements, all of which derive from mutations of nuclear genes: L 3000, uracil and histidine; L 4000, adenine,

uracil, and histidine; L 2200 and L 2300, adenine, tryptophan, and lysine; L 5628, adenine; L 2265, tryptophan, leucine, arginine, threonine and isoleucine.

Loss or Retention of Antibiotic Resistance in Petites

Respiratory competent, antibiotic resistant strains were grown for 18 h in yeast-extract–peptone–glucose medium containing 20 μg ethidium bromide/ml of medium. The cells were harvested, washed and crossed with an early stationary phase culture of an oligomycin-sensitive strain, and the diploid progeny from this cross analysed for resistance or sensitivity to the particular antibiotic as described.[26,27] It has been shown that such treatment with ethidium bromide yields petite (ρ^-) cultures whose cells lack mitochondrial DNA, symbolized ρ°.[28–30] Thus the loss of antibiotic resistance following this treatment, shown by the absence in the diploid progeny of the resistance characteristic of the ethidium bromide treated culture, indicates a cytoplasmic origin for the resistance mutation. Spontaneous ρ^- strains from resistant mutants were similarly examined for loss or retention of resistance characteristics by analysis of the diploid progeny of a cross with a sensitive strain.

Mitochondrial ATPase

Mitochondria were isolated from yeast cells grown on a 1% ethanol salts medium, as described previously[31] and purified on a 20–70% sucrose gradient. Mitochondrial ATPase was measured essentially as described by Somlo.[32] The reaction mixture (1 ml) contained the following: 50 mM tris-malate buffer (pH 6·2) or 50 mM tris-HCl (pH 9·4), 1 mM ATP, 1 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 17 enzyme units pyruvate kinase, and, where indicated, oligomycin dissolved in methanol over the range of concentrations given in the tables. The medium was preincubated for 5 min at 30° and the reaction was started by the addition of mitochondria (0·2–0·4 mg protein). After 10 min the reaction was stopped by the addition of 0·2 ml of a solution containing 0·8 M $HClO_4$ and 0·6 M Na_2SO_4, protein was removed by centrifugation, and inorganic phosphate was measured in 0·5 ml or 1·0 ml aliquots of the supernatant.[33]

As small amounts (5–10%) of non-mitochondrial ATPase were sometimes found to be present in the mitochondrial preparations, the ATPase activity in each strain was also measured in the presence of mitochondrial F_1 inhibitor, which specifically inhibits mitochondrial ATPase from yeast or mammalian cells and has no effect on non-mitochondrial ATPase. The F_1 inhibitor was prepared from beef heart mitochondria by the method of Pullman and Monroy.[34] The ATPase assay was slightly modified in the following way: F_1 inhibitor (200 μg/mg mitochondrial protein) was preincubated with mitochondria

for 10 min at 30°. A mixture, containing all other reaction components, was then added and the reaction was carried out for 10 min. The remainder of the procedure was exactly as described above. Results were corrected for any non-mitochondrial ATPase in the samples.

Specific activities are expressed as μmoles inorganic phosphate produced per min per mg mitochondrial protein.

Effect of Oligomycin on Anaerobically Grown Cells

Cells were grown anaerobically at 28°C for 22 h on a 5% glucose-1% Difco yeast extract-salts medium, in the presence or absence of the two lipid supplements, 0·5% Tween 80 and 20 mg/ml of ergosterol.[5] The cells were harvested at 0° and washed twice with sterile water.

Separate aliquots of the anaerobic cells were resuspended, at a concentration of 0·5–1·0 mg cells/ml medium in one of the following aeration media: 1% glucose–1% yeast extract-salts medium plus or minus oligomycin (10 μg/ml), (a concentration about 30 times greater than the amount required to prevent growth of sensitive strains on ethanol medium) or 1% ethanol-1% yeast extract–salts medium plus or minus oligomycin (10 μg/ml). Cells were aerated and samples were taken at 16 h to measure growth and cell viability. The respiration of washed whole cells was measured at 30° with an oxygen electrode in the presence of 50 mM potassium phosphate buffer (pH 7) and 2 mM glucose or 1% (w/v) ethanol. Whole cell cytochromes were measured on a Carey 14 recording spectrophotometer.

Protein synthesis. Assays of mitochondrial protein synthesis were carried out as previously described.[31]

Results

Mitochondrial ATPase Activity

Oligomycin is a potent inhibitor of both animal and plant mitochondrial ATPase. However while very low concentrations of oligomycin (about 1 μg oligomycin/mg mitochondrial protein) strongly inhibit ATPase in both animal and plant mitochondria, much higher levels are required to inhibit that of normal yeast mitochondria (about 20 μg oligomycin/mg mitochondrial protein). In wild type yeast mitochondrial ATPase is membrane bound and exhibits two pH maxima at 6·2 and 9·4;[35] ATPase activity at both pH's is inhibited by oligomycin. The *in vitro* effect of oligomycin on the mitochondria of two mutant strains of *S. cerevisiae* (L 3000, L 4000) resistant *in vivo* to oligomycin, and two characteristic sensitive strains (L 2200, L 410) are shown in Fig. 1. The two mutants L 3000 and L 4000 were selected for study as they differ in their genetic characteristics (see later). At both pH 6·2 and 9·4 neither of the mutant strains showed a clear pattern of *in vitro* resistance to oligomycin. Comparison of the ATPase system of

the sensitive strains with L 4000 shows small differences in sensitivity at pH 9·4 and 6·2, with the resistant mutant showing some increases in resistance over the range 5–40 μg of oligomycin. The mitochondrial ATPase of strain L 3000, on the other hand, shows essentially no difference in oligomycin sensitivity from that of the wild type strains at pH 9·4; however at pH 6·2 it is even more sensitive than them.

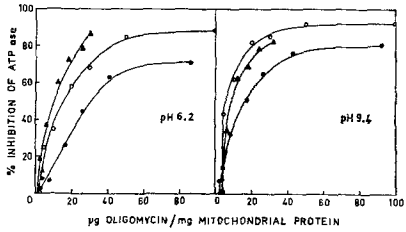

Figure 1. The effect of oligomycin on ATPase activity of isolated mitochondria. Mitochondria were isolated from ethanol grown cells and purified on a 20–70% sucrose gradient as described in Methods. Specific activities of mitochondrial ATPase, expressed as μmoles inorganic phosphate/min/mg mitochondrial protein were: strains L 410, L 2200, 1·35 (pH 6·2) and 1·96 (pH 9·4); strain L 4000, 0·58 (pH 6·2) and 1·47 (pH 9·4); strain L 3000, 1·01 (pH 6·2) and 1·12 (pH 9·4). ATPase activities in the presence of oligomycin are expressed as percentages of these controls. All values are corrected for non-mitochondrial ATPase. The ATPase of the oligomycin sensitive strains L 2200 and L 410 had almost identical *in vitro* activities in both the presence and absence of oligomycin (○—○). The results obtained with the mitochondria from the two oligomycin resistant strains are shown thus: L 3000, ▲—▲; L 4000, ●—●.

Anaerobic Growth and Aerobic Induction in the Presence of Oligomycin

The *in vivo* resistance and *in vitro* sensitivity of the oligomycin resistant mutants was reminiscent of previously studied mutants resistant to the mitochondrial protein synthesis inhibitors mikamycin and chloramphenicol; these were postulated to be mitochondrial membrane mutants.[20] Studies with these mutants showed that after anaerobic growth, during which the mitochondrial membranes are grossly altered, the resistant mutants could be rendered sensitive *in vivo* to mikamycin; they failed to develop respiration or the particulate mitochondrial cytochromes upon aeration in the presence of the drug. The experiments demonstrated that unlike some other mutants isolated in our laboratory,[25] the mitochondrial protein synthesizing system *per se* was not altered by the mutations, and that physiological alteration of membrane properties by anaerobic growth allows mikamycin to penetrate the altered membrane and inhibit mitochondrial protein synthesis, which in turn prevents cytochrome formation and the development of respiration.

The influence of anaerobic growth on oligomycin resistance was examined in a similar manner to that of mikamycin resistance. Yeast cells were grown anaerobically on lipid supplemented or lipid deficient media for 24 h and then aerated in either glucose or ethanol medium

in the presence and absence of oligomycin (see Methods section). The results were the same whichever type of anaerobically grown cells were employed. Both oligomycin sensitive and resistant strains aerated for 16 h on glucose medium, with or without oligomycin, are able to grow and develop a functional respiratory system and the particulate mitochondrial cytochromes (Table I). The extent of the development of

TABLE I. The effect of oligomycin on the growth and aerobic induction of respiration in anaerobically grown cells

Yeast strain	Aeration medium	Antibiotic addition	Aerobic induction: Cell density (mg/ml) To	Cell density (mg/ml) T_{16}	Whole cell respiration T_{16}	Cytochromes a, a_3, b, c_1 T_{16}
L 2200	Glucose	None	0·57	3·8	16	Present
	Glucose	OL	0·57	2·5	23	Present
	Ethanol	None	0·43	1·53	246	Present
	Ethanol	OL	0·43	0·43	76	Present
L 3000	Glucose	None	0·57	2·4	36	Present
	Glucose	OL	0·57	2·3	21	Present
	Ethanol	None	0·76	1·54	76	Present
	Ethanol	OL	0·76	0·76	13	Present
L 4000	Glucose	None	0·57	2·4	33	Present
	Glucose	OL	0·57	2·3	22	Present
	Ethanol	None	1·07	1·34	77	Present
	Ethanol	OL	1·07	1·07	19	Present

Cells were grown anaerobically on lipid supplemented medium for 24 h, harvested, washed and resuspended in ethanol or glucose aeration media in the presence or absence of oligomycin (10 μg/ml), see Methods. At the beginning of aeration (To), cell density was measured; these cells did not respire and the mitochondrial cytochromes a, a_3, b, and c_1 were absent. Cell density, whole cell respiration and cytochromes were measured after 16 h aeration. The effect of oligomycin (OL) on the strains *in vivo* under fully aerobic conditions, and *in vitro*, on the ATPase activity of isolated mitochondria, was as follows:

L 2200 *in vivo* and *in vitro*, oligomycin sensitive.
L 3000 *in vivo*, oligomycin resistant, *in vitro*, oligomycin sensitive.
L 4000 *in vivo*, oligomycin resistant, *in vitro*, oligomycin sensitive.

Whole cell respiration: mμ-atoms O_2/min/mg dry weight cells.

respiratory capacity on glucose media, due to catabolite repression, is limited. However it is sufficient to clearly show that in both resistant and sensitive cells, oligomycin does not inhibit the elaboration of the respiratory system or, in other words, inhibit mitochondrial protein synthesis. When the cells from either sensitive or resistant strains are aerated on ethanol, the cell mass increases and mitochondrial cytochromes and respiration develop. The extent of the growth of the organisms on glucose medium is considerably greater than on ethanol medium as the anaerobically grown cells can immediately extensively

utilize glucose for growth by non-oxidative pathways. On the other hand cells aerated on ethanol initially have no capacity to utilize ethanol. However both sensitive and resistant cells aerated on ethanol in the presence of oligomycin fail to grow but develop mitochondrial cytochromes and respiration.

It is well established that the mitochondrial membrane structure differs depending on whether the cells are grown anaerobically in the presence of an excess of unsaturated fatty acids and ergosterol or they are grown in the presence of growth limiting amounts of these lipids.[5, 8, 9] However, the alteration of the mitochondrial membrane properties as a consequence of anaerobic growth, irrespective of the presence of an added source of lipids, is extensive enough in both instances to allow oligomycin access to the oxidative phosphorylation system, and thus prevent derivation of energy from the respiratory chain and growth on ethanol.

Cross Resistance to Antibiotics

Previous work in this laboratory has shown that a number of cytoplasmically determined mutants are resistant to various combinations of inhibitors of mitochondrial protein synthesis, such as mikamycin, erythromycin, chloramphenicol, carbomycin, lincomycin, tetracycline and spiramycin and appear to be mitochondrial membrane mutants.[20] It was therefore of interest to examine possible cross-resistance patterns of oligomycin mutants to protein synthesis inhibitors. The two oligomycin resistance mutations can be readily distinguished one from the

TABLE II. Cross resistance in oligomycin resistant mutants

Yeast strain	Phenotype: *In vivo* / *In vitro*						
	Mikamycin	Chloramphenicol	Lincomycin	Carbomycin	Spiramycin	Erythromycin	Oligomycin
L 3000	R	R	R	R	S	S	R
	S	S	S	S	S	S	S
L 4000	S	S	S	S	S	S	R
	S	S	S	S	S	S	S

In vivo phenotypes, R (resistance) and S (sensitivity) denote the ability and inability, respectively, of cells to grow on ethanol medium containing the particular antibiotic, at concentrations as described in Methods.

In vitro phenotypes R and S denote resistance, defined as less than 25% inhibition of ^{14}C leucine incorporated into protein by isolated mitochondria, and sensitivity, defined as greater than 60% inhibition by 0·1 mM of the antibiotics mikamycin, chloramphenicol, lincomycin, carbomycin, spiramycin or erythromycin (see Methods).

In vitro resistance to oligomycin is defined as the lack of inhibition of ATPase from isolated mitochondria in the presence of the drug; sensitivity denotes 50% or more inhibition of mitochondrial ATPase by oligomycin (30 μg/mg mitochondrial protein).

other on the basis of their cross-resistance patterns so that the gene products involved are apparently not identical. The results are shown in Table II. The mutation to oligomycin resistance in strain L 4000 does not confer any cross-resistance to the antibiotics tested. On the contrary oligomycin resistance in strain L 3000 was simultaneously accompanied by cross-resistance *in vivo* to mikamycin, chloramphenicol, lincomycin, and carbomycin but not to spiramycin and erythromycin. However *in vitro* amino acid incorporation into protein by the isolated mitochondria from both L 3000 and L 4000 was inhibited by all the protein synthesis inhibiting antibiotics (Table II).

Inheritance Characteristics of the Oligomycin-resistant Mutants

The phenomenology of cytoplasmic genetics in yeast is still in an early stage of development. However some criteria have been suggested as descriptive of the process of cytoplasmic genetics.[1,20,25,36,37] The criteria are as follows: (a) on crossing a haploid wild type cell and a haploid mutant cell the diploid zygote formed contains both alleles of the cytoplasmic gene and these segregate during vegetative growth, to eventually give rise to a stable population of diploid progeny, some with the wild type characteristic and others with the mutant characteristic. This characteristic has been referred to as "mixedness" whereby a single zygote gives rise to a genetically mixed clone. The final ratio of stable resistant to sensitive diploid cells is a characteristic of each cross, but varies among individual zygotes. (b) On sporulation of purified diploid cells the cytoplasmic determinant segregates all or none into the four spores of each ascus. Thus in the case of an antibiotic resistant diploid cell sporulation gives rise to 4 resistant: O sensitive spores, and the converse is true for sensitive diploid cells. (c) The cytoplasmic gene may be deleted with ethidium bromide, a specific cytoplasmic mutagen. Indeed the drug has been shown to eliminate mitochondrial DNA from the cells without affecting cell viability.[29,30] The two classes of mutant isolated were examined for each of these criteria.

Characteristics of Diploid Progeny

Table III shows that when the oligomycin resistant strains L 4000 and L 3000 are crossed with several oligomycin sensitive strains L 2200, L 5628, L 2300 or L 2265, both stable oligomycin resistant and oligomycin sensitive diploids appeared in the diploid progeny from these crosses. Hence the cytoplasmic inheritance characteristic of mixedness of diploid progeny is observed with both these mutants.

Loss of Resistance with Petite Mutation

The elimination of the oligomycin resistance determinant from L 4000 by ethidium bromide treatment was demonstrated by growing

Table III. Mixedness characteristic of cytoplasmic inheritance

Cross			Diploid Progeny Oligomycin	
Oligomycin-R		Oligomycin-S	%R	%S
L 4000	×	L 2200	58	42
L 4000	×	L 5268	53	47
L 3000	×	L 2300	25	75
L 3000	×	L 2265	19	81

Cultures of each strain were crossed for 4 h, and diploids selected by prototrophic selection. Diploid progeny were assayed for oligomycin resistance (R) and sensitivity (S) as described in Methods: the degree of mixedness is reported as the percentage of oligomycin resistant and sensitive diploids in the total diploid progeny population examined, about 1000 cells per cross.

cultures overnight in the presence of the drug (20 μg/ml of medium), and subsequent crossing with untreated oligomycin sensitive strain L 2200. As a control, L 2200 was also treated similarly with ethidium bromide, and then crossed with untreated L 4000. The other oligomycin resistant strain L 3000, after treatment with ethidium bromide, was crossed with the untreated oligomycin sensitive strain L 2300; the control experiment was carried out similarly with ethidium bromide treated L 2300. The results are presented in Table IV and analysis of

TABLE IV. Loss of oligomycin resistance and sensitivity alleles on treatment with ethidium bromide

Ethidium bromide treated cells	Consequent cross	Diploid progeny
L 4000	L 4000 (ρ°) × L 2200 (ρ^{+}S)	All ρ^{+}S
L 2200	L 2200 (ρ°) × L 4000 (ρ^{+}R)	All ρ^{+}R
L 3000	L 3000 (ρ°) × L 2300 (ρ^{+}S)	All ρ^{+}S
L 2300	L 2300 (ρ°) × L 3000 (ρ^{+}R)	All ρ^{+}R

Cultures of oligomycin resistant (R) and sensitive (S) strains were treated with ethidium bromide (EtBr) at 20 μg drug/ml of medium for 18 h as described in Methods. Respiratory competent (ρ^{+}) cells are made respiratory deficient and lack mitochondrial DNA; such cells are designated ρ°.

Cultures of each haploid strain were crossed, and diploids selected and assayed for oligomycin resistance or sensitivity as described in Methods.

the zygotic progeny for resistance and sensitivity to oligomycin showed that only the characteristic of the untreated parent is expressed in the diploids from these crosses. The elimination of the determinant by ethidium bromide is thus evident.

Spontaneously arising petites from strains L 4000 and L 3000 were also examined for retention or loss of the oligomycin resistance determinant by crossing with oligomycin sensitive strains L 2200 and L 2300 respectively, and analysing diploid progeny for oligomycin resistance as before. A number of these spontaneously occurring petites of both resistant strains retained the resistance gene in contradistinction to the ethidium bromide treated cells. Eight out of 24 randomly selected petites of strain L 4000 retained the resistance gene, while 25 out of 48 petites of strain L 3000 similarly retained the resistance gene. Thus both these mutations to oligomycin resistance, in L 4000 and L 3000, are clearly linked to the classical cytoplasmic petite mutation.

Tetrad Analysis: Two Classes of Oligomycin Resistant Mutants

The foregoing data suggests that both classes of mutant are apparently cytoplasmic. However on examination of the segregation of oligomycin resistance in tetrads following meiosis, a different pattern of inheritance for each mutant becomes apparent; these are shown in Table V.

TABLE V. Tetrad analysis of oligomycin resistant mutants

Diploid/Cross	Resistance of diploid colony selected	No. of tetrads analysed	Segregation in spores Oligomycin Resistant:Sensitive
* N 1311	R	4	All 4:0
L 4000 × L 2200	R	5	All 4:0
	S	4	All 0:4
* N 1301	R	3	All 4:0
L 3000 × L 2300	R	13	All 2:2
	S	14	All 2:2

Strains N 1311 and N 1301 are oligomycin-resistant diploid mutants derived from the oligomycin-sensitive diploid N 1300 (see Methods). L 4000 is an oligomycin-resistant haploid strain derived from the sporulation of N 1311; L 3000 is similarly derived from N 1301. L 2300 and L 2200 aer oligomycin-sensitive strains. Cultures of each strain were crossed, and diploids selected as described in Methods. R and S denote oligomycin resistant and sensitive clones selected, sporulated and dissected as described in Methods. Nuclear alleles segregated 2:2 in all tetrads.

* Spore viability was poor in strains N 1311 and N 1301. However, random spore dissection yielded 72 spores from N 1311 and 66 spores from N 1301, all of which were oligomycin resistant.

When L 4000, an oligomycin resistant haploid derived from the oligomycin resistant mutant N 1311, was crossed with the oligomycin sensitive strain L 2200, mixed diploid progeny resulted. Sporulation of resistant diploids gave cytoplasmic 4:0 segregation in tetrads for resistance:sensitivity, and sensitive diploids gave the cytoplasmic 0:4 segregation. The diploid mutant N 1311 itself, when sporulated, showed 4:0 segregation in tetrads for oligomycin resistance to sensitivity. These

results clearly indicate the cytoplasmic character of the mutation to oligomycin resistance in strains N 1311 and L 4000.

On the other hand, the cross of strain L 3000 with L 2300 yielded mixed diploid progeny as described, but on sporulation, all tetrads examined showed nuclear 2:2 patterns of inheritance for oligomycin resistance to sensitivity whether from sporulated resistant or sensitive diploids (Table V). These tetrad data are inconsistent with the cytoplasmic inheritance characteristics of mixedness and ethidium bromide elimination of antibiotic resistance exhibited by strain L 3000. However it should be emphasized that direct sporulation of the oligomycin resistant mutant N 1301 from which L 3000 was derived shows 4:0 segregation in tetrads, indicative of cytoplasmic inheritance.

The oligomycin resistant strain L 3000 shows cross-resistance to the antibiotics mikamycin, chloramphenicol and carbomycin. Simultaneous examination of the segregation of these cross-resistances in diploids, and in all haploid spores from these diploids showed that they segregated with oligomycin resistance in all cases; also oligomycin sensitive diploids and spores were sensitive to all these antibiotics. Thus no separation of cross-resistances during vegetative growth, or following meiosis, was obtained. Similarly, all cross-resistances were lost simultaneously on ethidium bromide treatment. Spontaneously arising petites from L 3000 had retained or lost all drug resistances concomitantly. It appears that a single mutation in L 3000 had conferred simultaneously resistance to all these antibiotics.

A series of other mutants independently selected for oligomycin resistance have also been genetically analyzed. Four of these mutants behave identically to N 1311 and L 4000, and three behave in the same way as N 1301 and L 3000.

Discussion

The mitochondria of cells of the two representative mutants L 3000 and L 4000 are resistant to oligomycin; in addition the mitochondria of L 3000 are cross resistant *in vivo* to the protein synthesis inhibiting antibiotics mikamycin, chloramphenicol, lincomycin and carbomycin whereas L 4000 is sensitive to these drugs. These results establish a clear biochemical difference between the two mutants. However the resistance characteristics of the mitochondria have not to date been demonstrated *in vitro*; mitochondria isolated from strains L 3000 and L 4000 are sensitive to oligomycin and all the other antibiotics. The ATPase and OSCP do not therefore appear to be the site of either of the two mutations. Indeed, that these two protein complexes are not directly affected by the mutations is further shown by the demonstration that growth of the two oligomycin resistant mutants under anaerobic conditions renders both types of cells sensitive to oligomycin inhibition. Clearly the alteration of the mitochondrial membrane properties

produced by anaerobic growth results in the basically sensitive ATPase complex being accessible to oligomycin.

The results indicate that the mitochondrial membrane during isolation of the organelles is physically modified and hence the mitochondrial ATPase and protein synthesizing system are rendered accessible to inhibition by oligomycin and the protein synthesis inhibiting antibiotics. Similarly, physiological modification of the mitochondrial membranes by anaerobic growth renders the organelle membranes permeable to each of the antibiotics. The data are interpreted to indicate that the two mutations affect a protein (*s*) of the mitochondrial membrane.

The mutation to oligomycin resistance in L 4000 indicates the exclusion of this drug from access to the mitochondrial ATPase; it might therefore be described as a simple mitochondrial membrane mutation specifically affecting the permeability of the organelle to oligomycin. One possible candidate for the likely altered membrane protein of the mitochondria of strain L 4000 may be that recently described by Subik *et al.*[38] and Tzagaloff[39] which appears to be required for the binding of mitochondrial ATPase and OSCP to the electron transport system. However explanations of this kind are probably too simplistic when the properties of L 3000 are considered.

The observation that in strain L 3000 both the mitochondrial protein synthesizing system and the ATPase complex are shielded *in vivo* from oligomycin and some chemically unrelated antibiotics (mikamycin, chloramphenicol, lincomycin, carbomycin) but not others (erythromycin, spiramycin) suggests a more complicated situation may be being observed. It may be considered that the membrane alteration in L 3000 is such that conformational changes are induced which simultaneously shield the ATPase complex from oligomycin and partly shield the mitochondrial ribosome (which is attached to the mitochondrial membrane[2,40]) from some antibiotics, but not others.[20] This interpretation is supported by the isolation in our laboratory of other mutants with a wide variety of cross resistance patterns to antibiotics herein under examination; these mutants are then envisaged as membrane mutations inducing conformational membrane changes which in turn lead to different extents of ATPase and ribosome shielding.[1,2,40,41] Thus the attachment of the mitochondrial ribosome to the membrane provides one rationale for different single mutations producing such complex cross-resistance patterns among the protein synthesis inhibitors and the correlation that apparently a single mutation can in addition confer oligomycin resistance. It must be recalled that many of the antibiotics are chemically unrelated and those drugs which are chemically related, do not necessarily group in cross-resistance patterns. Further, chemically related compounds such as carbomycin, spiramycin and erythromycin react at different ribosomal

sites,[42–44] so a membrane conformational change could conceivably screen one or more of the reaction sites, leaving the others exposed. Thus all antibiotics could *in vivo* penetrate the mitochondrial organelle, but dependent upon the conformational screening of the ribosome only some antibiotics could react with the ribosomes.

The inheritance characteristics of strain L 4000 are entirely consistent with the phenomena associated with cytoplasmic inheritance outlined in Results. Hence, this cytoplasmically determined mitochondrial mutant resembles, in its genetic properties, the previously described cytoplasmic mutants, resistant to erythromycin,[25] paromomycin[45] and chloramphenicol.[37] These mutants are still biochemically poorly characterized. The erythromycin mutation has been shown to involve a change in the mitochondrial protein synthesizing system,[46] apparently in the mitochondrial ribosome.[47] The mutation to paromomycin resistance also appears to be expressed as a change in the mitochondrial protein synthesizing system[45] while the chloramphenicol mutants have not been biochemically investigated.

The other mitochondrial membrane mutant L 3000 simultaneously shows characteristics of both nuclear and cytoplasmic inheritance. In crosses between resistant and sensitive strains, it shows the cytoplasmic inheritance pattern of the production of mixed diploid progeny arising from single primary zygotes. A cytoplasmic association of oligomycin resistance is also shown by both its retention or loss in cytoplasmic petites. Only following crosses between oligomycin resistant and sensitive cells and subsequent meiosis is a nuclear inheritance characteristic observed, namely as a nuclear 2:2 segregation ratio of 2 OL^r:2OL^s in tetrads. During the processes of cytoplasmic segregation, meiosis and petite formation the antibiotic cross-resistance pattern (oligomycin, mikamycin, chloramphenicol, carbomycin, lincomycin) shows no separation, all the antibiotic resistances stay together. The mutation to multiple antibiotic resistance in L 3000 appears to be a single mutation.

In a study of a tetracycline resistant mutant, Wilkie[45] has reported that on sporulation of sensitive and resistant diploids, 4 sensitive:0 resistance spores and 2 sensitive:2 resistant spores respectively are obtained. Also he has reported that there is no association between the tetracycline mutation and the rho factor.[45] These observations are clearly different to those reported herein but also involve some type of nucleo-cytoplasmic genetic interaction.

Both cytoplasmic and nuclear patterns of inheritance have been separately reported for oligomycin resistant mutants.[1, 21–24, 48] However the present data should not be confused with those previously reported, the results clearly establish both nuclear and cytoplasmic genetic behaviour interacting in the one mutant. It is not established as yet whether the primary mutation is cytoplasmic or nuclear.

However it is clear that the rho factor determines the phenotype of the cell. Thus in a cross involving one cell which has been converted to ρ° (mit-DNA absent) it is the other ρ^{+} cell in the cross which determines whether the diploids formed will be resistant or sensitive to the antibiotic. Yet on sporulation of the resultant ρ^{+} diploids whether sensitive or resistant, 2 sensitive and 2 resistant haploid spores are obtained, which in subsequent crosses can vegetatively transmit the apparent cytoplasmic characteristic of sensitivity or resistance. Any simple concept of dominant or recessive nuclear alleles does not appear to account for the observations. Little is known of the fate of the mitochondrial DNA during sporulation; such knowledge now appears to be crucial to a full understanding of cytoplasmic genetic phenomena in yeast. It may be that possibly we are observing part of a global phenomenon, whereby all cytoplasmic genomes be they virus determining, bacterial sex or R factors, mitochondrial DNA, chloroplast DNA and as yet uncharacterized DNA's of the endoplasmic reticulum and cell surface etc. are of a nature that they can all on occasion, be integrated into the nuclear genome and thus all have some properties similar to the classical bacterial episome.

An alternative and more attractive hypotheses can be advanced. Consider that the primary mutation to resistance is nuclear and that the gene product (a mitochondrial membrane component) requires an appropriate mitochondrial membrane conformation for its acceptance into the membrane; the phenotypic expression of the gene product then becomes possible. The apparent cytoplasmic inheritance would then be envisaged as being determined by the transmission and segregation of preformed resistant or sensitive mitochondrial membranes among progeny cells. In heterozygotes formed in crosses between sensitive and resistant ρ^{+} cells the pre-existing membrane conformations would then play their essential roles in the progeny by determining whether it is the nuclear gene product of the sensitive or resistant allele which is inserted into developing mitochondrial membrane. It is envisaged in this hypothesis that the mutation to petite leads to loss or retention of resistance as determined by the extent of the membrane deformation caused by the petite mutation rather than a direct effect of the ρ^{-} mutation on a cytoplasmic oligomycin resistance gene. It follows that resistance retention in the petite mutants requires the mitochondrial membrane structure to be still capable of accepting the nuclear resistance gene product; loss of resistance means that the particular petite mutation has altered the mitochondrial membrane to such an extent that it cannot accept the nuclear resistance gene product. The membranes have, in this hypothesis, a degree of self determination. These interpretations owe much to the insight and concepts developed by Sonneborn to explain the heredity of the cortical patterns of *Paramecium*.[49]

A preliminary study of some of the genetic properties of strain L 3000 in relation to mikamycin resistance has been reported earlier.[20] At the time it appeared that the mutations to antibiotic resistance in strain L 3000 and several other strains (L 3200, L 3300) were classical cytoplasmic mutations analogous to the erythromycin resistance mutation.[25] However it is clear from the results reported herein that the situation is not so simple and that nucleo-cytoplasmic interactions are occurring in strain L 3000. The genetic characteristics of strain L 3000 are probably not unique but are representative of a considerable number of mutants recently isolated in our laboratory which are phenotypically characterized by a variety of cross resistance patterns different to that of L 3000. A full account of this work is being prepared for publication elsewhere.[41]

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