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BIOGENESIS OF MITOCHONDRIA

oli2 MUTATIONS AFFECTING THE COUPLING OF OXIDATION TO PHOSPHORYLATION IN *SACCHAROMYCES CEREVISIAE* *

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Summary

1. Two oligomycin-resistant strains of *Saccharomyces cerevisiae* have been isolated and shown to have mutations in the *oli2* region of the mitochondrial DNA. On solid media containing a non-fermentable energy source, the mutant strains were able to grow only slowly at 28°C and not at all at 18°C or 36°C.

2. When grown in a glucose-limited chemostat at 28°C, the mutant strains were almost completely defective in oxidative metabolism. The mutant mitochondria contained significant levels of all respiratory enzymes, and an active, oligomycin-sensitive ATPase, but the ATP-$^{32}P_i$ exchange activity and P : O ratio were very low.

3. The mutations in these strains are genetically closely linked to *mit*$^-$ mutations which have been shown to affect a 20 000-dalton ATPase subunit (Roberts, H., Choo, W.M., Murphy, M., Marzuki, S., Lukins, H.B. and Linnane, A.W. (1979) FEBS Lett. 108, 501—504). Since the mitochondrial ATPase in these mutant strains appears to be fully assembled, the defect in the coupling mechanism is probably a result of a small alteration in the structure of the 20 000-dalton ATPase subunit.

4. When the mutant strains were grown at 18°C, the mitochondria had very low cytochrome oxidase activities, and reduced levels of cytochrome aa_3. The largest subunit (M_r 40 000) of this enzyme was not synthesized.

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Introduction

The key to the elucidation of the process of oxidative phosphorylation is an understanding of the energy-transducing mechanism of the mitochondrial APTase (ATP phosphohydrolase, EC 3.6.1.3). ATPase preparations from several eukaryotic sources have been described, but the composition of the enzyme complex is still only poorly defined. The yeast oligomycin-sensitive ATPase complex contains at least nine protein components, of which five form a catalytic complex denoted F_1 [1,2]. The remaining subunits constitute the membrane sector (F_0) of the ATPase complex, and are concerned with energy coupling. It has been suggested that as many as four of the subunits of the membrane sector are synthesised on mitochondrial ribosomes [3]. However, recent evidence from our laboratory (Ref. 4 and Orian, J.M., Murphy, M., Marzuki, S. and Linnane, A.W., unpublished data) as well as elsewhere [5] suggests that only two subunits of the enzyme complex, with molecular weights of 7600 and about 20 000, are synthesised in the mitochondrion.

The structure and function of the membrane sector can be studied by isolating and analysing yeast strains carrying mitochondrial mutations which specifically affect the ATPase. Such mutations have been mapped in two distinct regions of the mitochondrial genome, designated *oli1* and *oli2*. The *oli1* region is located between 18 and 20 map units on the mitochondrial DNA [6], and has been shown to contain the structural gene of the 7600-dalton ATPase proteolipid subunit [7—9]. The *oli2* region (34—36 map units [10]) is separated from the *oli1* region by a large segment of mitochondrial DNA at least 10 000 base-pairs long. Several mutations in the *oli2* region (class C mutations [6]) have been shown to confer resistance to oligomycin on cells growing on non-fermentable substrates. The mitochondrial ATPase in these strains exhibits weak resistance to oligomycin in intact mitochondria and in Triton extracts of mitochondria [11]. Several mutations with a mit^- phenotype have also been mapped in the *oli2* region and shown to result in the loss of oxidative phosphorylation [12,13]. We have recently reported that some of these mit^- mutations specifically affect the 20 000-dalton subunit of the mitochondrial ATPase, and are therefore probably in the structural gene of this subunit [13,14].

In this communication we describe a new class of *oli2* oligomycin-resistant mutants which, as the result of single mutations, are also partially defective in oxidative metabolism. The evidence shows that the 20 000-dalton subunit of the ATPase complex is necessary for energy coupling between the respiratory chain and the ATPase.

Several laboratories have reported that mutations within the *cob-box* region of the mitochondrial DNA, which contains the structural gene for cytochrome *b*, can affect the synthesis of cytochrome oxidase subunit I [15—18]. However, this effect is not limited to mutations in this region of the mitochondrial genome: mutations in the *var1* and *oli1* regions can also affect the synthesis of cytochrome oxidase subunit I (Ref. 19 and Murphy, M., unpublished data). A similar effect is now described in the new *oli2* mutants. When the growth temperature is lowered, these mutant strains are also unable to synthesise subunit I of cytochrome oxidase.

Materials and Methods

Strains and mutant isolation. The mutant strains C58 and 803 discussed in this paper were derived from the oligomycin-sensitive wild-type strain J69-1B α *ade1 his* [*rho*$^+$]. The wild-type parent was first mutagenized with Mn^{2+} according to Putrament et al. [20] and then plated for selection of resistant clones on agar (1.5%) medium containing yeast extract (0.5%), peptone (1%), ethanol (2% v/v), and oligomycin (2 μg/ml). Strains J69-2C a *ura lys2* [*rho*$^+$] and L2200 a *ade1 lys2 trp1* [*rho*$^+$] are wild-type strains. Strain 823 α *ade1 his* [*rho*$^+$ *oli23-r*] is an oligomycin-resistant mutant derived from strain J69-1B [21], and strain 70M a *ade1 lys2 trp1* [*rho*$^+$ *oli23-r*] is a spore clone from a cross between strains 823 and L2200. The oligomycin-resistant strain D22/A15 [22] and the *oli2 mit*$^-$ strains Ma1, Mb12 and Ma30 [13] have been described previously. Strain EJO is a *rho*$^\circ$ derivative of J69-1B obtained by ethidium bromide-elimination of mtDNA.

Growth of cells. Except where indicated, all yeast strains were grown in glucose-limited chemostat cultures under catabolite-derepressed conditions as previously described [23]. For cells growing at 28°C the dilution rate was 0.1 h^{-1}, and for 18°C growth the dilution rate was 0.05 h^{-1}. For batch growth, cells were grown aerobically in 50-ml side-arm flasks on a rotary shaker in 20 ml of medium containing yeast extract (1% w/v), mineral salts [24] adenine (100 μg/ml), histidine (50 μg/ml), and ethanol (1% v/v). Cell growth was estimated by measuring the absorbance of the culture in a Klett-Summason colorimeter.

Isolation and analysis of mitochondria. Mitochondria were routinely isolated according to the method of Roberts et al. [25], and published procedures were used to assay ATPase [13], NADH-cytochrome *c* reductase [26] and cytochrome *c* oxidase activities [27]. For studies of coupling activities, mitochondria were isolated according to the method of Cobon et al. [28] with the addition of bovine serum albumin (2 mg/ml) before the protoplasts were disrupted and in all subsequent steps. The ATP-P_i exchange procedure of Ryrie [29] was used, except that 0.25 M sucrose was added to the reaction mixture, dithiothreitol was not included, and the incubation temperature was 30°C. Oxidative phosphorylation was measured according to Schatz [30], except that ubiquinone and cyanide were not added. Inorganic phosphate was extracted by the method of Avron [31].

Labelling of mitochondrial translation products. Cells were labelled with [^{35}S]sulphate in the presence of cycloheximide (a specific inhibitor of cytoplasmic protein synthesis) as previously described [21], except that the incubation times were doubled for cells grown at 18°C. Mitochondria were isolated from the cells [25] in the presence of protease inhibitors [32], and samples of these mitochondria were analysed by slab gel electrophoresis as previously described [21].

Antibody precipitation of ATPase. [^{35}S]Sulphate-labelled mitochondria were prepared as above and the ATPase was extracted by suspending these mitochondria in 5 mM Tris acetate (pH 7.5) containing 0.5% (w/v) Triton X-100 and protease inhibitors as above, at a protein concentration of 6.25 mg/ml. After 10 min this solution was centrifuged at 100 000 $\times g$ for 20 min. To the supernatant an equal volume of rabbit antiserum raised against purified oligo-

mycin-sensitive ATPase [33] was added, and precipitation was allowed to proceed overnight. The precipitate was collected by centrifugation at 12 000 × *g* for 30 s and washed three times in the buffer used for extraction. Samples of these precipitates were analysed by slab gel electrophoresis as above.

Results

Strains C58 and 803 carry mutations in the oli2 region of mtDNA

Mutants C58 and 803 were obtained as independent isolates during a screening for oligomycin-resistant mutants. In addition to oligomycin resistance, isolates C58 and 803 exhibited two further phenotypic characteristics. They were able to grow only slowly on a non-fermentable energy source at 28°C, and were unable to utilise non-fermentable substrates at either 18°C or 36°C. The growth of these strains on fermentable substrates was not affected at any of these temperatures.

The mutant characteristics were shown to be cytoplasmically-inherited by a variety of genetic tests. For example, in crosses of both strains C58 and 803 to wild-type strains, the mutant and wild-type phenotypes segregated during mitosis of the zygotes. Other evidence includes cytoplasmic transfer of the mutant phenotypes to haploid strains carrying the *kar 1-1* mutation [34] and elimination of the mutant determinants by treatment of strains C58 and 803 with ethidium bromide.

No segregation of the three mutant characteristics (oligomycin-resistance, partial defect in oxidative metabolism at 28°C, and temperature-conditional respiratory deficiency) was observed when the mutant strains were crossed with two wild-type strains (Table I). This indicates that the pleiotropic phenotype arose either by single mutations in the mtDNA, or by closely linked multiple mutations.

The mutations in strains C58 and 803 were shown to lie in the *oli2* locus of the mitochondrial genome by crossing the mutants with a standard tester strain carrying a class C oligomycin-resistance mutation [6] in the *oli2* region, as shown in Table II. The *oli23-r* mutation in strains 70M and 823 is very close or allelic to the original O_{II} mutation in strain D22/A15 described by Avner and

TABLE I

TRANSMISSION OF OLIGOMYCIN RESISTANCE, PARTIAL DEFECT IN OXIDATIVE METABOLISM, AND TEMPERATURE-CONDITIONAL RESPIRATORY DEFICIENCY IN CROSSES OF *oli2* MUTANT STRAINS

Strains were crossed by conventional techniques. The diploid progeny were tested for their sensitivity to oligomycin and their ability to grow on media containing ethanol at 28, 18 and 36°C. Strains J69-2C and L2200 are oligomycin-sensitive and grow normally on ethanol at 18, 28 and 36°C.

Cross	Total colonies scored	Phenotypic classes		
		Wild-type	Mutant parental	Other
C58 × J69-2C	597	309	288	0
C58 × L2200	216	130	86	0
803 × J69-2C	578	388	190	0
803 × L2200	559	229	230	0

TABLE II

LINKAGE OF MUTATIONS IN *oli2* MUTANT STRAINS TO *oli2* OLIGOMYCIN-RESISTANCE MUTATIONS

The *oli2* temperature-conditional and oligomycin-resistant strains were mated on agar media by conventional techniques. The *mit*$^-$ strains were mated with strain 70M in 2 ml of liquid medium containing yeast extract (1%), peptone (1%) and glucose (10%). After 6 h, nutrients were washed out, and the cells were innoculated into 5 ml of minimal medium containing adenine and glucose (10%). After standing for 2 days, the cells were reinnoculated into fresh minimal medium containing adenine and glucose. After standing for a further 1 day, the diploids were spread onto plates containing minimal medium, adenine and glucose (2%), and replica plated onto plates containing yeast extract, peptone and ethanol (2%), and onto plates containing yeast extract, peptone, ethanol and oligomycin (2 μg/ml). Strains D22/A15, 823 and 70M carry *oli2* oligomycin resistance mutations. Strains Ma1, Mb12 and Ma30 carry *mit*$^-$ mutations mapping in the *oli2* region.

Cross	Ethanol-positive diploid colonies	Oligomycin-sensitive recombinant diploids	Percentage of oligomycin-sensitive diploids
D22/A15 × 823	557	0	0
C58 × 70M	636	0	0
803 × 70M	1652	0	0
Ma1 × 70M	827	1	0.12
Mb12 × 70M	1211	9	0.74
Ma30 × 70M	1228	9	0.73

Griffiths [22,35]. In crosses of strains C58 and 803 with strain 70M, no oligomycin-sensitive recombinants were detected. Likewise, tight linkage was also observed in crosses of strain 70M to a group of *mit*$^-$ strains carrying mutations at the *oli2* locus which have been shown specifically to affect the synthesis of the 20 000-dalton protein component of the mitochondrial ATPase [13]. The recombination mapping of the mutations of strains C58 and 803 at the *oli2* locus was confirmed by petite mapping studies: only petite mutants retaining the *oli2* locus were able to complement the respiratory deficient phenotype of C58 and 803 at 18°C or 36°C to produce respiratory competent diploid progeny.

Strains C58 and 803 are defective in oxidative metabolism

The *oli2* mutants which were used in this study grow only slowly in batch

TABLE III

GROWTH OF WILD-TYPE AND *oli2* MUTANT STRAINS AT 28°C

Cells were grown in batch cultures with 1% (v/v) ethanol as the carbon source, or in glucose-limited chemostat cultures with a dilution rate of 0.1 h^{-1}. The glucose concentration of the inflowing medium was 1%, and that of the outflowing medium was less than 0.002%. Petite frequencies of strains C58 and 803 were 49% and 38%, respectively.

Strain	Batch cultures		Chemostat cultures
	Generation time (h)	Y_{EtOH} (g cell dry wt./mol ethanol)	Y_{glu} (g cell dry wt./mol glucose)
J69-1B	4.8	29	100
C58	9.5	29	29
803	14	26	32
EJO	—	0	24

cultures containing non-fermentable energy sources at the permissive growth temperature of 28°C (Table III). However, the final growth yields of the mutant strains are similar to that of the wild-type, which indicates that despite the slow growth the mutant strains can still obtain the same amount of energy from the non-fermentable energy source as the parent strain J69-1B, but less rapidly than the parent.

The defect in oxidative metabolism at 28°C becomes more apparent when the mutant strains are grown in glucose-limited chemostat cultures. Under these growth conditions, the steady state growth yields of both mutants (Table III) are greatly decreased compared with the wild type. Indeed, the growth yields are only slightly higher than that of a mtDNA-less *petite* (ρ^0) strain of yeast, which completely lacks oxidative phosphorylation. The chemostat was operated at a dilution rate of 0.1 h^{-1}, which imposes a generation time of 7 h on each strain. Thus the *oli2* mutant cells divide more frequently in the chemostat culture than they do in batch cultures, and it is likely that the process of assembly of a component of the oxidative phosphorylation system becomes rate-limiting.

ATPase and respiratory activities of strains C58 and 803

The nature of the biochemical defect responsible for the inability of the mutant strains to grow by oxidative metabolism in chemostat cultures was investigated. Although the mutations in these strains are located in the *oli2* region of the mitochondrial genome, which has previously been shown to contain the loci of mutations affecting the ATPase [11—13], the inability of strains C58 and 803 to grown on non-fermentable energy sources is not due to a deficiency in ATPase activity in the mutant strains (Table IV). Not only do the mitochondria contain significant levels of ATPase activity, but this activity is also quite sensitive to high levels of oligomycin. Thus, although the concentration of oligomycin which causes 50% inhibition of the ATPase activity in mitochondria of strain C58 is 28 μg/mg protein, compared with 10 μg/mg protein in the wild type, the maximal degree of inhibition is the same in both

TABLE IV

ATPase AND RESPIRATORY ACTIVITIES IN MITOCHONDRIA OF WILD-TYPE AND *oli2* MUTANT STRAINS GROWN AT 28°C

The strains were grown in chemostat cultures as described in Table III, and mitochondria were isolated. Petite frequencies of strains C58 and 803 were 35% and 38%, respectively. The concentrations of inhibitors used were 20 μg oligomycin per ml, 1.7 μg antimycin per ml, and 3 mM KCN.

Strain	ATPase		NADH cytochrome c reductase		Cytochrome oxidase	
	Specific activity (μmol/min per mg)	Oligomycin sensitivity (% inhibition)	Specific activity (μmol/min per mg)	Antimycin sensitivity (% inhibition)	Specific activity (μmol/min per mg)	Cyanide sensitivity (% inhibition)
J69-1B	0.91	86	0.37	90	0.40	98
C58	0.69	91	0.16	90	0.12	65
803	0.99	51	0.21	89	0.08	80

TABLE V

RESPIRATION OF WILD-TYPE AND *oli2* MUTANT CELLS GROWN IN CHEMOSTAT CULTURE AT 28°C

Whole cell respiration was measured polarographically at 30°C in 2.5 ml of 50 mM phosphate buffer (pH 7.0). The cell concentration was 1 mg dry wt./ml and the reaction was started by the addition of 10 µl of absolute ethanol. The concentration of CCCP was 50 µM, and that of KCN was 1.2 mM. The petite frequencies of strains C58 and 803 were 35% and 38%, respectively.

Strain	Respiration rate (nmol O_2/min per mg cell dry wt.)		
	No addition	+CCCP	+KCN
J69-1B	59	123	0
C58	83	70	6
803	59	60	2

strains. This indicates that all of the ATPase in these mitochondria is in the form of an F_1-F_0 complex.

The respiration of wild-type and mutant cells grown at 28°C in chemostat cultures is shown in Table V. Despite the lower growth yields, the respiration rates of the mutant cells are equal to or greater than that of the wild-type cells. Mitochondria isolated from the mutant strains contain all of the respiratory cytochromes (Fig. 1), and significant although somewhat reduced respiratory

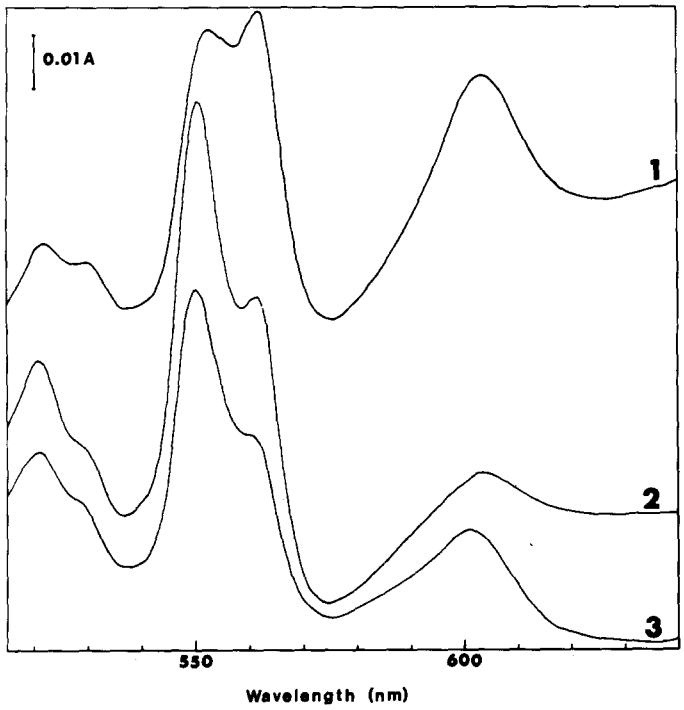

Fig. 1. Absorption spectra of mitochondria isolated from wild-type and *oli2* mutant cells grown in chemostat culture at 28°C. Mitochondria were isolated and resuspended at a protein concentration of 7.5 mg/ml in 0.6 M sorbitol containing 0.7 mM EDTA and 13 mM Tris-HCl (pH 7.4). Mitochondria were reduced with sodium dithionite and oxidised with ammonium persulphate, and difference spectra were recorded with an Aminco DW 2a spectrophotometer. 1, strain J69-1B; 2, strain 803; 3, strain C58.

enzyme activities (Table IV). These apparently reduced levels are, with the possible exception of the cytochrome oxidase activity in strain 803, due to the large percentage of respiratory-deficient petite cells in the cultures of both mutant strains. The relatively high respiratory activities of the mutant strains in vivo, together with the normal ATPase activities of the mitochondria, suggest that the defect in these *oli2* mutants is probably at the level of coupling of oxidative phosphorylation. This is supported by the observation that the chemostat cultures of the mutant strains contained very low steady-state concentrations of ethanol.

Oxidative phosphorylation is uncoupled in the oli2 mutants

The *oli2* mutants were screened for lack of coupling by observing the response of the cells to an uncoupler, CCCP (Table V). It was found that CCCP rapidly penetrated the cells of the wild-type strain and stimulated the respiration about 100%, presumably due to the release from respiratory control. Thus this method provides a means of rapid screening of mutations that cause uncoupling in yeast. When the mutant cells were analysed by this method, the uncoupler was found to have no stimulatory effect. The results indicate either that oxidative phosphorylation is already uncoupled in the mutants, or that they have an altered uncoupler binding site.

In order to characterise further the nature of the biochemical lesion in the *oli2* mutants, a more detailed analysis of coupling was carried out in one of these mutants, strain C58. Table VI shows the results of measurements of oxidative phosphorylation and ATP-$^{32}P_i$ exchange performed on mitochondria isolated from wild-type and C58 cells grown at 28°C in chemostat cultures. The wild-type mitochondria have a P : O ratio of 1.5, which is close to the theoretical ratio of 2 for yeast mitochondria. However, in strain C58 the P : O ratio is 0.18, only 10% of the wild-type ratio. The uncoupling of this strain is reflected by its low mitochondrial ATP-$^{32}P_i$ exchange activity, which is about 10% of the wild-type activity. These results clearly show that the ATPase in this strain is almost completely unable to synthesise ATP.

TABLE VI

OXIDATIVE PHOSPHORYLATION AND ATP-P_i EXCHANGE ACTIVITY OF WILD-TYPE AND C58 MITOCHONDRIA

The strains were grown in chemostat cultures at 28°C and intact mitochondria were isolated [28]. P:O ratios were measured as mol of ATP synthesised per mol of NADH oxidised. The concentration of oligomycin was 20 μg/ml, and that of CCCP was 250 μM, where indicated. The culture of strain C58 contained 30% petite cells.

Strain	Additions to assay	P:O ratio	ATP-P_i exchange (nmol/min per mg)
J69-1B		1.5	160
J69-1B	oligomycin	0.34	0
J69-1B	CCCP	0.00	0
C58		0.18	12
C58	oligomycin	0.14	1
C58	CCCP	0.09	0

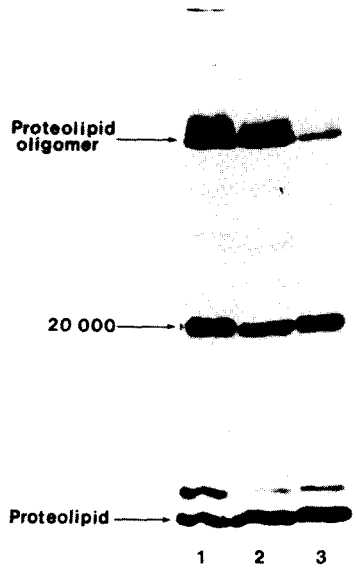

Fig. 2. Mitochondrial translation products precipitated by anti-ATPase antisera in wild-type and mutant strains. [^{35}S]Sulphate-labelled mitochondria were prepared from cells grown in chemostat cultures at 28°C, and antibody precipitates, prepared using an antibody directed against the oligomycin-sensitive ATPase [33], were analysed in polyacrylamide slab gels as described in Materials and Methods. Above are displayed immunoprecipitated mitochondrial products from cells of strain J69-1B (1), strain 803 (2), and strain C58 (3). 20 000 indicates the position of the 20 000-dalton ATPase subunit.

Assembly of ATPase complex in oli2 mutants

We have previously reported that some *mit*$^-$ mutations in the *oli2* region resulted in the absence of the 20 000-dalton subunit of the ATPase complex, presumably due to the premature termination of translation leading to the production of shorter polypeptides [13,14]. However, the following analysis shows that this is not the case in the *oli2* mutants C58 and 803. The products of mitochondrial protein synthesis were labelled at 28°C with $^{35}SO_4^{2-}$ in the presence of cycloheximide, and the ATPase was immunoprecipitated using an antiserum to the oligomycin-sensitive ATPase complex. The radioactively-labelled proteins associated with the immunoprecipitated ATPase complex were analysed by electrophoresis in polyacrylamide slab gels in the presence of sodium dodecyl sulphate (Fig. 2). In the wild-type strain there are four major bands, with molecular weights of 7600 (the proteolipid), 9500 (probably not a component of the ATPase [4,5]), 20 000 and 50 000 (an oligomer of the proteolipid [36]). The immunoprecipitated ATPase of strains C58 and 803 appears to have an identical pattern to the wild-type, indicating that none of the ATPase subunits synthesised in the mitochondria is absent when these strains are grown at 28°C.

The lack of gross abnormalities in the mitochondrially synthesised subunits of the ATPase of the mutants indicates that the uncoupling of oxidative phosphorylation and the low ATP-P_i exchange activities in these strains are not due to an inability of the cells to synthesise any of the components of the mitochondrial ATPase. It is likely that the mutations cause a small modification,

TABLE VII

LACK OF RESPIRATORY ACTIVITY IN STRAINS C58 AND 803 WHEN GROWN AT 18°C

Growth rates were determined in batch cultures with 1% (v/v) ethanol as the energy source. Other experiments were carried out on cells grown in glucose-limited chemostat cultures at a dilution rate of 0.05 h^{-1}. Cellular respiration rates were measured as described in the legend of Table V. The stimulation of respiration was measured on addition of 50 μM CCCP. There was no significant cyanide-insensitive respiration. Mitochondrial cytochrome oxidase activities were at least 98% sensitive to 1.2 mM cyanide. The NADH-cytochrome *c* reductase activities were inhibited at least 84% by 1.7 μg antimycin per ml.

	Strain		
	J69-1B	C58	803
Whole cells			
Generation time (h)	8.5	28	—
Respiratory activity (nmol O_2/min per mg dry wt.)	59	10	3
Respiratory activity in presence of CCCP (nmol O_2/min per mg dry wt.)	125	12.5	3
Isolated mitochondria			
Cytochrome oxidase (μmol/min per mg)	0.45	0.015	0.016
NADH-cyt *c* reductase (μmol/min per mg)	0.22	0.20	0.14
ATPase (μmol/min per mg)	1.20	0.42	0.39
ATPase activity in presence of 20 μg oligomycin per ml (μmol/min per mg)	0.13	0.09	0.09

such as an amino acid substitution, in the protein encoded in the *oli2* region. This alteration is not detectable by polyacrylamide gel electrophoresis, but is sufficient to impair the function of the ATPase, resulting in the uncoupling of oxidative phosphorylation.

Defect in cytochrome oxidase in oli2 mutants grown at 18°C

In addition to the uncoupling of oxidative phosphorylation observed in cells of strains C58 and 803 grown at 28°C, the *oli2* mutations in these strains have a secondary effect which can only be observed when they are grown at 18°C (Table VII). At this temperature, the strains exhibit a respiratory deficiency which can best be demonstrated in batch cultures; at 18°C with ethanol as the energy source, strain 803 does not grow at all, and strain C58 grows about three times more slowly than at 28°C (compare Table VII with Table III).

The growth yields of the mutant strains in glucose-limited chemostat cultures at 18°C were very much reduced compared with that of the wild type. Unlike the mutant cells grown at 28°C, the respiration rates of the mutant cells grown at 18°C are only about 10% of the rates observed in wild-type cells (Table VII). This respiration is not significantly stimulated by uncouplers. Mitochondria isolated from the mutant strains grown at 18°C have very low cytochrome oxidase activities. The NADH-cytochrome *c* reductase and ATPase activities are not markedly affected by the decreased growth temperature, although they are somewhat reduced in strain 803 (compare Tables IV and VII). The loss of cytochrome oxidase activity in the mutant strains is reflected by a concomitant loss of the cytochrome aa_3 absorption band in isolated mitochondria (Fig. 3).

In order to determine the nature of the lesion in cytochrome oxidase in the mutant cells grown at 18°C, proteins synthesised in the mitochondria of the

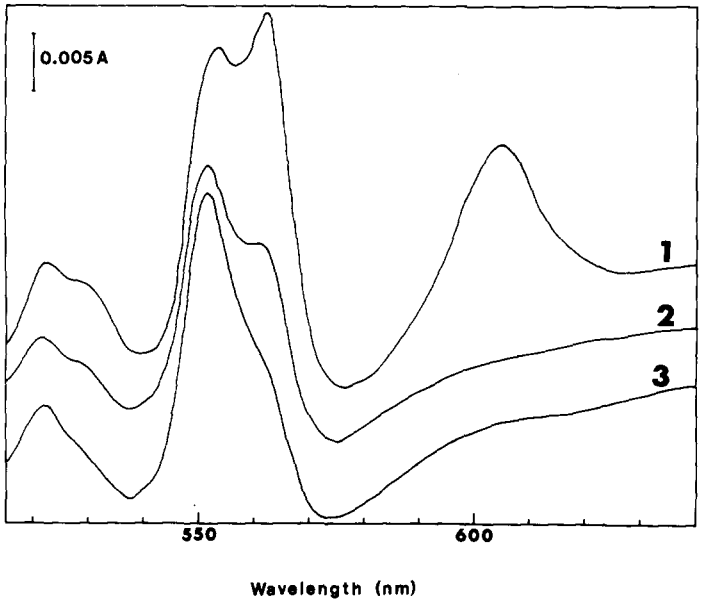

Fig. 3. Absorption spectra of mitochondria isolated from wild-type and *oli2* mutant cells grown in chemostat culture at 18°C. Reduced vs. oxidised spectra were recorded as in Fig. 1. 1, strain J69-1B; 2, strain 803; 3, strain C58.

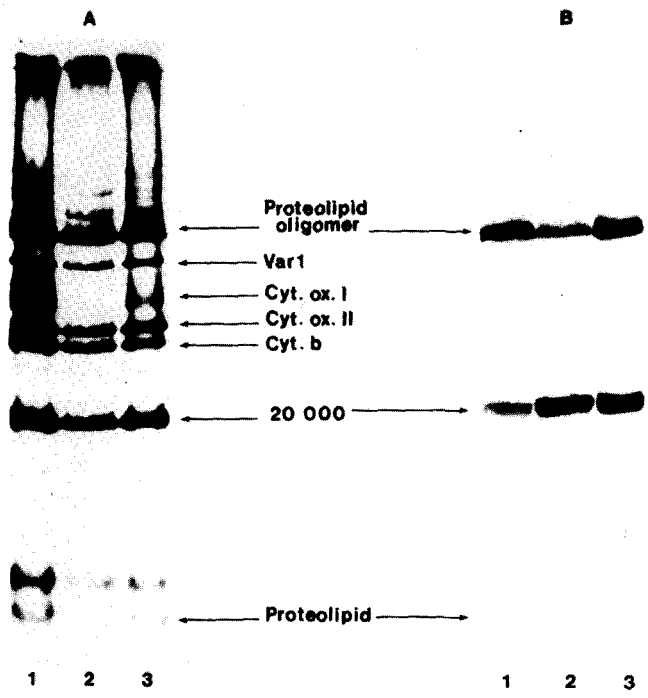

Fig. 4. Mitochondrial translation products in whole mitochondria and in ATPase immunoprecipitates from wild-type and *oli2* mutant strains grown at 18°C. Cells were grown in chemostat cultures and labelled with [^{35}S]sulphate in the presence of cycloheximide, and mitochondria were isolated as in Materials and Methods. A. Samples of these mitochondria were electrophoresed on polyacrylamide slab gels. Above are displayed mitochondrial products from strain J69-1B (1), strain 803 (2), and strain C58 (3). B. ATPase was immunoprecipitated using an antibody directed against the oligomycin-sensitive ATPase complex [33] and analysed in polyacrylamide slab gels. Above are displayed immunoprecipitated mitochondrial translation products from strain J69-1B (1), strain 803 (2), and strain C58 (3). The bands labelled cyt. ox. I and II represent subunits I and II of cytochrome oxidase; cyt. *b* is the cytochrome *b* apoprotein; the 20 000-dalton band is composed of the 20 000-dalton ATPase subunit, and, in whole mitochondria, subunit III of cytochrome oxidase.

mutant strains were labelled with ^{35}S at 18°C and analysed by electrophoresis on sodium dodecyl sulphate polyacrylamide gels (Fig. 4a). Under these conditions, cytochrome oxidase subunit I (M_r 40 000) is absent in strain 803 and there is very little synthesis of this subunit in strain C58. There are no obvious deficiencies in any of the other mitochondrial translation products in the mutant strains. In particular, the immunoprecipitated ATPase of the mutants displayed the same pattern of mitochondrial translation products as the wild-type strain (Fig. 4b).

Discussion

Previous studies in our laboratory, showing that *mit*$^-$ mutations in the *oli2* region affect the 20 000-dalton subunit of the membrane sector in the ATPase, suggest that the *oli2* region contains the structural gene for this subunit [13,14]. In the *oli2* mutant strains C58 and 803, the subunit is not grossly altered or lacking, and the activity and oligomycin-sensitivity of the ATPase in these strains indicates that the assembly of the ATPase is not grossly impaired by the mutations. Therefore, the mutations probably cause only a small modification of the 20 000-dalton subunit, which then either cannot be precisely orientated in the ATPase complex, or is unable to function in the normal way. These *oli2* mutants are similar in some ways to one of the *pho1* mutants reported by Foury and Tzagoloff [12], and studied by Somlo and Krupa [37]. However, the *pho1* mutant appears to have contained an unstable ATPase, which resulted in a partial loss of oxidative phosphorylation and a partial defect in the attachment of F_1 to the membrane in isolated mitochondria.

While strains C58 and 803 have a coupled oxidative phosphorylation system when grown in batch cultures at 28°C, they are able to obtain energy only slowly from ethanol. When the cells are forced to grow at the same rate as the parent strain in glucose-limited chemostat cultures, the metabolic defect becomes more apparent. Under these conditions, the mutant cells oxidise most of the ethanol (produced by fermentation), but cannot utilize the energy released. The ability of the cells to consume ethanol, without significantly increasing the cell mass, indicates that the mitochondria are defective in oxidative phosphorylation in vivo, and the low level of oxidative phosphorylation observed in isolated mitochondria is not due to an instability of the ATPase. The low P : O ratios and ATP-P_i exchange activities of the mitochondria indicate that the mitochondria lack a functional link needed for the coupling of ATPase activity to the energy conserving mechanism, which may be a transmembrane proton gradient. It is possible that the 20 000-dalton subunit of the membrane sector of the ATPase is specifically involved in the generation, maintenance or utilisation of the proton gradient linked to the synthesis or hydrolysis of ATP.

In addition to the defect in coupling at 28°C, when the mutant strains were grown under derepressed conditions at 18°C, a deficiency in the synthesis of cytochrome oxidase subunit I became evident. Such interactions between mitochondrial genes, whereby a mutation in one gene (for example the structural gene for cytochrome *b*) can affect the production of another protein (e.g. cytochrome oxidase subunit I) encoded in a separate region of the mitochondrial

DNA, have been observed previously, but their mechanisms are unknown [15—19,21]. However, this is the first reported incidence of the loss of cytochrome oxidase subunit I in a mutant without the loss of the gene product which is primarily affected by the mutation. It is possible that these two defects which are the result of a single mutation in each strain, are not unrelated, but reflect an underlying sequence in the assembly of the enzyme complexes of the inner membrane. Such a sequence is also implied in the order of appearance of the enzymes of the mitochondrial inner membrane during respiratory adaptation of catabolite-repressed yeast cells [38,39]. The synthesis of cytochrome oxidase, which is the last enzyme to appear, may well be prevented as a result of a mutation affecting a component of one of the first complexes to be assembled, the ATPase.

An alternative explanation of the loss of cytochrome oxidase subunit I has previously been proposed [4]. The *oli2* mutations map in a region adjacent to the *cya2* region of the mitochondrial DNA. The latter region is extremely large (occupying about 20% of the mitochondrial genome) and probably carries the structural gene for subunit I of cytochrome oxidase [40,41]. Some large species of RNA isolated from yeast mitochondria have been found to hybridise to restriction fragments of mitochondrial DNA which contain both the *oli2* and *cya2* regions of the genome [42]. It is likely that this RNA is an mRNA precursor, and is transcribed as a single piece covering this region. These *oli2* mutations may cause a defect in the processing of this mRNA precursor, resulting in the observed pleiotropic effect. Pleiotropic effects of mutations in other regions of the mitochondrial DNA [15—19,21], may also be due to a defect in the processing of large mRNA precursors. In support of this idea, preliminary evidence has recently been obtained in our laboratory that the mitochondrial DNA is transcribed as large RNA species, which might even cover the entire mitochondrial genome [4].

References

1 Takeshige, K., Hess, B., Böhm, M. and Zimmermann-Telschow, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1605—1622
2 Ryrie, I.J. and Gallagher, A. (1979) Biochim. Biophys. Acta 545, 1—14
3 Tzagoloff, A. and Meagher, P. (1972) J. Biol. Chem. 247, 594—603
4 Linanne, A.W., Marzuki, S., Nagley, P., Roberts, H., Beilharz, M., Choo, W.M., Cobon, G.S., Murphy, M. and Orian, J.M. (1980) in Proceedings of the Fourth John Innes Symposium, The Plant Genome, and Second International Haploid Conference (Davies, D.R. and Hopwood, D.A., eds.), pp. 99—110, The John Innes Charity, Norwich, U.K.
5 Enns, R. and Criddle, R.S. (1977) Arch. Biochem. Biophys. 183, 742—752
6 Trembath, M.K., Molloy, P.L., Sriprakash, K.S., Cutting, G.J., Linnane, A.W. and Lukins, H.B. (1976) Mol. Gen. Genet. 145, 43—52
7 Sebald, W., Wachter, E. and Tzagoloff, A. (1979) Eur. J. Biochem. 100, 599—607
8 Macino, G. and Tzagoloff, A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 131—135
9 Hensgens, L.A.M., Grivell, L.A., Borst, P. and Bos, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1663—1667
10 Choo, K.B., Nagley, P., Lukins, H.B. and Linnane, A.W. (1977) Mol. Gen. Genet. 153, 279—288
11 Griffiths, D.E. and Houghton, R.L. (1974) Eur. J. Biochem. 46, 156—167
12 Foury, F. and Tzagoloff, A. (1976) Eur. J. Biochem. 68, 113—119
13 Roberts, H., Choo, W.M., Murphy, M., Marzuki, S., Lukins, H.B. and Linnane, A.W. (1979) FEBS Lett. 108, 501—504
14 Stephenson, G., Marzuki, S. and Linnane, A.W. (1980) Biochim. Biophys. Acta 609, 329—341
15 Claisse, M.L., Spyridakis, A., Wambier-Kluppel, M.L., Pajot, P. and Slonimski, P.P. (1978) in Biochemistry and Genetics of Yeasts (Bacila, M., Horecker, B.L. and Stoppani, A.O.M., eds.), pp. 369—390, Academic Press, New York

16 Alexander, N.J., Vincent, R.D., Perlman, P.S., Miller, D.H., Hanson, D.K. and Mahler, H.R. (1979) J. Biol. Chem. 254, 2471—2479
17 Hanson, D.K., Miller, D.H., Mahler, H.R., Alexander, N.J. and Perlman, P.S. (1979) J. Biol. Chem. 254, 2480—2490
18 Haid, A., Schweyen, R.J., Bechmann, H., Kaudewitz, F., Solioz, M. and Schatz, G. (1979) Eur. J. Biochem. 94, 451—464
19 Linnane, A.W., Astin, A., Marzuki, S., Murphy, M. and Smith, S. (1979) in Extrachromosomal DNA: I.C.N.-U.C.L.A. Symposia on Molecular and Cellular Biology (Cummings, D.J., Borst, P., Dawid, I.B., Weissman, S.M. and Fox, C.F., eds.) Vol. 15, pp. 249—267, Academic Press, New York
20 Putrament, A., Baranowska, H. and Prazmo, W. (1973) Mol. Gen. Genet. 126, 357—366
21 Murphy, M., Choo, K.-B., Macreadie, I., Marzuki, S., Lukins, H.B., Nagley, P. and Linnane, A.W. (1980) Arch. Biochem. Biophys. 203, in the press
22 Avner, P.R. and Griffiths, D.E. (1973) Eur. J. Biochem. 32, 301—311
23 Marzuki, S. and Linnane, A.W. (1979) Methods Enzymol. 56, 568—577
24 Wallace, P.G., Huang, M. and Linnane, A.W. (1968) J. Cell Biol. 37, 207—220
25 Roberts, H., Choo, W.M., Smith, S.C., Marzuki, S., Linnane, A.W., Porter, T.H. and Folkers, K. (1978) Arch. Biochem. Biophys. 191, 306—315
26 Hatefi, Y. and Rieske, J.S. (1967) Methods Enzymol. 10, 225—231
27 Wharton, D.C. and Tzagoloff, A. (1967) Methods Enzymol. 10, 245—250
28 Cobon, G.S., Crowfoot, P.D. and Linnane, A.W. (1974) Biochem. J. 144, 265—275
29 Ryrie, I.J. (1975) Arch. Biochem. Biophys. 168, 704—711
30 Schatz, G. (1967) Methods Enzymol. 10, 30—33
31 Avron, M. (1960) Biochim. Biophys. Acta 40, 257—272
32 Ryrie, I.J. (1977) Arch. Biochem. Biophys. 184, 464—475
33 Tzagoloff, A. and Meagher, P. (1971) J. Biol. Chem. 246, 7328—7336
34 Nagley, P. and Linnane, A.W. (1978) Biochem. Biophys. Res. Commun. 85, 585—592
35 Avner, P.R., Coen, D., Dujon, B. and Slonimski, P.P. (1973) Mol. Gen. Genet. 125, 9—52
36 Tzagoloff, A. and Akai, A. (1972) J. Biol. Chem. 247, 6517—6523
37 Somlo, M. and Krupa, M. (1979) Mol. Gen. Genet. 167, 329—335
38 Mahler, H.R., Perlman, P.S. and Mehrotra, B.D. (1971) in Autonomy and Biogenesis of Mitochondria and Chloroplasts (Boardman, N.K., Linnane, A.W. and Smillie, R.M., eds.), pp. 492—511, North Holland Publishing Co., Amsterdam
39 Kim, I.-C. and Beattie, D.S. (1973) Eur. J. Biochem. 36, 509—518
40 Cabral, F., Solioz, M., Rudin, Y., Schatz, G., Clavilier, L. and Slonimski, P.P. (1978) J. Biol. Chem. 253, 297—304
41 Eccleshall, T.R., Needleman, R.B., Storm, E.M., Buchferer, B. and Marmur, J. (1978) Nature 273, 67—70
42 Grivell, L.A., Arnberg, A.C., Boer, P.H., Borst, P., Bos, J.L., Van Bruggen, E.F.J., Groot, G.S.P., Hecht, N.B., Hensgens, L.A.M., Van Ommen, G.J.B. and Tabak, H.F. (1979) in Extrachromosal DNA: I.C.N.-U.C.L.A. Symposia on Molecular and Cellular Biology (Cummings, D.J., Borst, P., Dawid, I.B., Weissman, S.M. and Fox, C.F., eds.), Vol. 15, pp. 305—324, Academic Press, New York