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OXIDATIVE PHOSPHORYLATION IN YEAST

VI. ATPase ACTIVITY AND PROTEIN SYNTHESIS IN MITOCHONDRIA ISOLATED FROM NUCLEAR MUTANTS DEFICIENT IN CYTOCHROMES

J. ŠUBÍK, Š. KUŽELA, J. KOLAROV, L. KOVÁČ AND *T. M. LACHOWICZ

*Department of Biochemistry, Komensky University, Bratislava (Czechoslovakia) and *Institute of Immunology and Chemotherapy, Polish Academy of Sciences, Wroclaw (Poland)*

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SUMMARY

1. The oligomycin-sensitive Mg^{2+}-dependent ATPase activity of mitochondria isolated from wild-type yeast *Saccharomyces cerevisiae* was only slightly inhibited by atractyloside at concentrations which entirely prevented oxidative phosphorylation. This indicated that most of the ATPase in these mitochondrial preparations was located outside the atractyloside-sensitive barrier and did not participate in the energy-transfer process.

2. ATPase activity of mitochondria isolated from nuclear gene mutants deficient in a single cytochrome, *a*, *b*, or *c*, respectively, was strongly inhibited by oligomycin. The mitochondria from these mutants, like those from the wild-type strain, were able to incorporate amino acids into protein.

3. Mitochondrial ATPase activity of single nuclear gene mutants deficient in both cytochromes *a* and *b* was only slightly inhibited by oligomycin. These mitochondria were incapable of incorporating amino acids into protein. The mitochondria from these nuclear mutants thus resembled mitochondria of cytoplasmic respiration-deficient mutants.

4. The results suggest that mitochondrial cytochromes may be coded by nuclear genes and that product(s) of mitochondrial protein synthesis may be required for integrating the cytochromes *a* and *b* and the components of the oligomycin-sensitive ATPase complex into the mitochondrial membranes.

INTRODUCTION

An increasing number of biochemical mutants with modified mitochondria substantiates the recent proposals for using mutants in a stepwise investigation of oxidative phosphorylation[1,2]. This approach, although superficially similar to that employed in the study of intermediary metabolism, has some unique features when applied to membrane-linked processes. Unity of structure and function in biological membranes may limit a simple sequential analysis of chemical events, since some

modifications may destroy the entire system. Owing to the cooperativity in membranes[3] and to the allotopic properties of mitochondrial enzymes[4], a lesion in a single step may be spread over the entire system and affect other steps as well.

With this proviso in mind, mitochondrial ATPase activity, which is generally considered a part of the energy transfer system, has been studied in different yeast mutants exhibiting a modified complement of respiratory pigments. These lesions were correlated with the ability of isolated mitochondria to incorporate amino acids into protein.

EXPERIMENTAL

A diploid wild-type strain of *Saccharomyces cerevisiae* DT XII was already employed in a previous study[5]. Haploid nuclear gene mutants were prepared by exposing a haploid strain of *S. cerevisiae* D 225-5 A (α ad_1 lys_2), originating from Centre de génetique moléculaire du C.N.R.S., Gif-sur-Yvette, to mutagenesis with ultraviolet light or NO_2^-. Their isolation and genetic characteristics will be described in more detail elsewhere[6]. Haploid strains were either used as such or converted to diploids homozygous for the affected nuclear gene. The fraction of cytoplasmic ρ^- mutants in the haploid strains was evaluated by crossing a haploid strain with a neutral cytoplasmic ρ^- mutant of opposite mating type, isolating zygotes either by micromanipulation or by prototrophic selection and testing them by growth on noncarbohydrate substrates and by the triphenyltetrazolium overlay technique[7].

The methods of culture and of isolation of mitochondria as well as enzyme determination and analytical techniques were the same as employed previously[5]. Amino acid incorporation by isolated mitochondria was assayed by the procedure of LAMB *et al.*[8].

Atractyloside was a gift from Dr. T. Galeotti (Catholic University, Rome) and oligomycin from Upjohn Co. Antimycin A was purchased from Sigma and other chemicals from Lachema.

RESULTS

Additional properties of mitochondrial ATPase from wild-type yeast

The ATPase activity of wild-type yeast mitochondria was found previously[5] to display two pH optima. At pH optimum 6.2 it was strongly inhibited by F^- and only slightly by N_3^- and oligomycin; an inverse sensitivity to the inhibitors was observed at the second pH optimum of 9.5.

As shown in Table I, ATPase activity in wild-type yeast mitochondria was essentially insensitive to inhibition by CN^- and antimycin A. More surprisingly, it was only slightly inhibited by atractyloside, an inhibitor of adenine nucleotide translocation across the mitochondrial membrane[9], even though, in a parallel experiment, atractyloside at a concentration of 100 μg/mg mitochondrial protein inhibited oxidative phosphorylation by 96.5%. It might be assumed that the exposure of mitochondria to pH 9.5 had destroyed the atractyloside-sensitive barrier; however, even at pH 7.5 and 6.2 (not shown in the table) the ATPase was only slightly sensitive toward atractyloside.

TABLE I

INHIBITION OF ATPASE ACTIVITY IN WILD-TYPE YEAST MITOCHONDRIA

The reaction mixture contained in 1.0 ml: 4 mM ATP, 80 mM KCl, 0.5 mM EDTA, 20 mM Tris–HCl buffer, 80 mM mannitol, 2 mM $MgCl_2$ and mitochondria (0.05 mg protein) from aerobically grown *S. cerevisiae* DT XII. Final pH and inhibitors used are indicated in the table. All the inhibitors except CN^- were added as methanolic solutions; final concentration of methanol was 1%; 30°, 10 min. The values are means of two experiments.

Inhibitor	*Concn.*	*Inhibition* (%)	
		pH 7.5	*pH 9.5*
CN^-	1 mM	0	0
Antimycin A	10 μg/ml	—	10.0
Oligomycin	40 μg/ml	—	93.6
Atractyloside	20 μg/ml	3.4	12.4
Atractyloside	100 μg/ml	12.5	20.8

Biochemical characteristics of nuclear gene mutants deficient in cytochromes

It has been found by genetic tests that in each of the mutants employed in this study only a single nuclear gene (*p*, see ref. 7) was affected. Since nuclear gene mutations leading to respiratory deficiency in yeast are often accompanied by an increased mutability to the cytoplasmic ρ^- genotype, resulting in double $p\rho^-$ mutants[7,10], the fraction of such double mutants in the culture of haploid cells was determined. It was necessary to carry out such determinations several times in the course of this study in order to exclude the possibility that some properties typical of the ρ^- genotype were erroneously ascribed to nuclear gene mutants. In general, the fraction of double $p\rho^-$ cells in the haploid cultures studied did not exceed 40%.

The mutants lacking cytochrome *a*, or *a* and *b* simultaneously, displayed values of $-Q_{O_2}$ on glucose lower than 1. Diploid homozygous strains deficient in either cytochrome *b* or cytochrome *c* had $-Q_{O_2}$ values on glucose of 39 and 48, respectively, the corresponding value for the wild-type strain being 146. Q_{CO_2} on glucose in air with all strains was in the range of 270–360. Typical cytochrome spectra of the strains are shown in Fig. 1. Strains Z 8, S 10 and S 18 contained no spectrally discernable

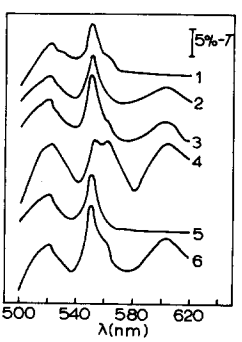

Fig. 1. Difference spectra of yeast mutants. Cells were suspended in an equal volume of water and their spectra recorded in the SF 10 spectrophotometer. Cells in the sample cuvette were reduced by endogenous substrate(s) and in the reference cuvette oxidized by 60 mM H_2O_2. 1, cytochrome *a*-deficient strain S 18, haploid culture; strains Z 8 and S 10 exhibited a similar spectrum; 2. cytochrome *b*-deficient strain 981-9 D, haploid culture; 3, homozygous diploid strain 981-9 D; 4, cytochrome *c*-deficient strain S 14, diploid culture; 5, cytochromes *a*- and *b*-deficient strain Z 5; strains S 5, S 9 and S 19 exhibited similar spectra; 6, wild-type strain D 225-5 A.

cytochrome *a*, in both their haploid and diploid states. In the spectrum of the haploid strain 981-9 D the presence of cytochrome *b* was dubious; in the corresponding diploid cultures cytochrome *b* was certainly present, albeit in a smaller amount than in wild-type yeast. Possibly, cytochrome *b* synthesis was not entirely blocked in this mutant but only slowed down, so that two doses of the mutated gene in a cell may have increased the rate of cytochrome *b* synthesis.

Strains S 14 showed a modified spectrum in the cytochrome *c* region. Cytochrome *c* was either completely absent or substantially reduced, so that the presence of cytochrome c_1 became prominent. The mutant may be related to *cy* mutants studied by other investigators[11,12].

The phenotypic properties of strains Z 5, S 5, S 9 and S 19 are identical with those of cytoplasmic respiration-deficient mutants[13,14] lacking both cytochromes *a* and *b*.

ATPase activity of the mutant mitochondria

Only the activity at pH 9.5 was extensively studied, but it was verified with several strains that the mutant mitochondria displayed ATPase activity with two pH optima, at 6.2 and 9.5, as does the wild-type strain[5] and a cytoplasmic respiration-deficient mutant[15]. The specific activity at pH 9.5 was lower than in wild-type yeast and was similar to that of cytoplasmic mutants. The lower specific activities may be due to catabolic repression, which appears to be generally stronger in mutant yeasts with impaired respiratory ability[16].

The properties of the mutant ATPases are listed in Table II. The most important conclusion to be drawn from these data is that mitochondrial ATPase of nuclear mutants deficient in a single cytochrome is highly sensitive to oligomycin, whereas the ATPase from nuclear mutants deficient in both cytochromes *a* and *b* is rather

TABLE II

MITOCHONDRIAL ATPASE ACTIVITY OF NUCLEAR CYTOCHROME-DEFICIENT MUTANTS

Conditions similar to those in Table I. The values are means of two or three experiments. The strains with crosses between identical marks represent homozygous diploids. Oligomycin was added as a methanolic solution; final concentration of methanol was 1 %. Specific activity is expressed in μmoles $P_i \cdot min^{-1} \cdot mg^{-1}$.

Deficient cytochrome	*Strain*	*Specific activity*	*Inhibition of ATPase* (%)			
		Oligomycin ($\mu g/ml$):	*35*	*5*	*1*	*0**
a	Z 8	0.88	76.0	74.4	76.0	10.2
	S 10 × S 10	0.87	78.2	74.0	74.0	2.0
	S 18 × S 18	1.17	75.5	74.7	75.7	6.2
b	681-9 D	2.12	70.0	70.0	68.0	8.0
c	S 14 × S 14	1.95	90.0	88.0	82.0	10.0
a + *b*	Z 5	1.32	27.5	27.5	27.5	7.5
	S 5	1.38	22.0	17.0	14.0	17.0
	S 9	1.90	25.0	23.0	18.2	5.0
	S 9 × S 9	0.81	14.5	7.0	15.0	6.0
	S 19 × S 19	1.10	17.0	4.5	8.0	0

* Methanol alone.

insensitive to this inhibitor and thus resembles the ATPase of cytoplasmic respiration-deficient mutants[15,17].

Protein synthesis by isolated mutant mitochondria

As shown in Table III, mitochondria from the wild-type strain as well as from mutants deficient in a single cytochrome were able to incorporate amino acids into protein. The incorporation ability was entirely absent from mitochondria of nuclear mutants deficient in both cytochromes *a* and *b*. A similar lack of protein synthesis was previously found in mitochondria isolated from cytoplasmic respiration-deficient mutants[18,19] and corroborated by incorporation experiments *in vivo*[20].

TABLE III

INCORPORATION OF [^{14}C]LEUCINE BY ISOLATED MITOCHONDRIA OF NUCLEAR CYTOCHROME-DEFICIENT MUTANTS

The reaction mixture contained in 1.0 ml: 40 μmoles Tris–HCl buffer (pH 7.4), 5 μmoles KH_2PO_4, 100 μmoles KCl, 8 μmoles $MgCl_2$, 1 μmole ATP, 10 μmoles phosphoenolpyruvate, 30 μg pyruvate kinase, 0.4 μC [^{14}C]leucine (24 μC/μmole) and approx. 1 mg of mitochondrial protein; 20 min, 30°.

Deficient cytochrome	*Strain*	*Leucine incorporation (pmoles · 20 min^{-1} · mg^{-1})*
o (Wild type)	DT XII	72
a	Z 8	84
	S 18 × S 18	75
b	681-9 D	82
c	S 14 × S 14	74
a + *b*	Z 5	<2
	S 9 × S 9	<2
	S 19	<2

DISCUSSION

The ATPase activity of wild-type yeast mitochondria has been found to be strongly inhibited by oligomycin but only moderately by atractyloside, even though both inhibitors completely inhibited oxidative phosphorylation in these mitochondria. To account for this observation it is pertinent to remember that "intact" yeast mitochondria, as prepared by us as well as in other laboratories[1,5,21,22], always exhibited a high Mg^{2+}-dependent ATPase activity, the rate of which at pH 9.5 is almost an order of magnitude higher than the rate of oxidative phosphorylation[5]. On the other hand, the ATPase activity of the more familiar rat-liver mitochondria is essentially latent and activated by Mg^{2+} only after destroying mitochondrial integrity (*e.g.* ref. 23). It is reasonable to assume that most of the yeast mitochondrial ATPase is modified by the procedure used in the preparation of the mitochondria so that it is accessible to ATP from the outside of the atractyloside-sensitive barrier, although the oligomycin-sensitivity is maintained. Apparently only that small part of the ATPase system that is inhibited by atractyloside is involved in the energy transfer mechanism proceeding inside the atractyloside-sensitive barrier.

Nuclear gene mutations resulting in mitochondria deficient in a single cytochrome apparently do not affect the ATPase system. Mitochondria isolated from these mutants have also preserved their ability to incorporate amino acids into protein. At least some of the genes involved in these mutations may be structural genes for the particular cytochrome. The fact that mutants with such deficiencies are nuclear and that they can carry on mitochondrial protein synthesis as do cells containing the complete assembly of the cytochromes suggests that all the cytochromes may be coded for by nuclear DNA. This has been proved previously in the case of iso-1-cytochrome *c* (ref. 12).

On the other hand, several independent nuclear gene mutations resulting in the absence of both cytochromes *a* and *b* from mitochondria also affect the energy transfer system, since the ATPase activity of such mutants is no longer sensitive to oligomycin. Since cytoplasmic mutation to respiratory deficiency similarly leads to loss of cytochromes *a* and *b* and to damage of the oligomycin sensitivity-conferring system[15,17], most rigorous measures were taken to exclude the possibility that the present results were simply due to a cytoplasmic mutation which was superimposed upon the nuclear mutation. The evaluations of contamination with double $p\rho^-$ mutants were carried out independently in two laboratories and by two methods. Obviously, even the highest figures obtained with some strains (40 % of double $p\rho^-$ mutants) could not account for reduction in oligomycin sensitivity to the levels virtually identical with those found previously in pure ρ^- cultures[15,17,19].

Both the nuclear and the cytoplasmic mutants with the complex deficiencies were not able to carry on mitochondrial protein synthesis. The absence of mitochondrial protein synthesis in the two classes of the mutants may be a common basis of their deficiencies. The product of mitochondrial protein synthesis may be required to assemble[14,24–26] or to hold in proper configuration cytochromes *a* and *b* (and probably also cytochrome c_1, see ref. 14) and also oligomycin sensitivity-conferring components in the mitochondrial membrane. Alternatively, some components of the complex oligomycin sensitivity-conferring system[27–29] may be synthesized inside the mitochondria and represent the assembling factor(s) for the cytochromes.

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