ADP-DEPENDENT THERMAL REACTIVATION OF TRITON-INACTIVATED ATPASE FROM MITOCHONDRIALLY DETERMINED OLIGOMYCIN-RESISTANT MUTANTS OF *SACCHAROMYCES CEREVISIAE*.

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SUMMARY. Triton X-100, employed during purification of the mitochondrial ATPase complex (7), did not significantly affect ATPase activity from the oligomycin-sensitive wild type, while the ATPase activity from two mitochondrially determined oligomycin-resistant mutants was strongly inhibited. During subsequent thermal treatment, the wild type ATPase was protected by ATP or ADP, while the ATPase from the mutants was reactivated under the same conditions. The existence of a relationship between oligomycin-sensitivity, Triton-inhibition and the mitochondrially synthesized subunits of the ATPase complex is suggested.

Yeast mitochondrial ATPase complex[§](1) has been described as composed of 9 types of subunits. F_1 (catalytic subunits (2)) and OSCP (necessary but insufficient for oligomycin-sensitivity (3)), are synthesized on cytoplasmic ribosomes, wheras several other subunits (part of the "membrane factor") are synthesized on mitoribosomes. Although the respective functional role of the latter subunits is not yet known, one or several of them are necessarily implied in oligomycin-sensitivity (1). It was previously shown that the mitochondrial ATPase from two oligomycin-resistant strains mutated at either of the mitochondrial loci O_I or O_{II} (4) was relatively resistant to oligomycin (5,6), presumably due to a modification in part of the "membrane factor". This paper describes a genetically determined change in some general properties of the ATPase complex, revealed by the study of ADP-dependent thermal reactivation of Triton-inactivated ATPase.

MATERIAL AND METHODS

Chemicals : described in (6). Strains : the three isonuclear strains PS 194 (O^S), PS 195 (O^R_{144} mutated at the locus O_{II} of mitochondrial DNA) and PS 211 (O^R_{146} mutated at the locus O_I of mt-DNA) were described in (6). Aerobic glucose culture medium and harvesting as in (7). Cells, washed in TSE buffer (Tris-Cl 50 mM, pH 7.2, Sorbitol 0.6 M, EDTA 0.25 mM), were suspended in 30 ml TSE (total 1.5-2.0 g cell proteins) for disruption in a homogenizer (B. Braun, Melsungen) with 20 g glass beads (diameter 0.45 mm) at 4000 osc./min for 30 sec. Subcellular fractionation and purification : scheme 1. The

[§] ATPase complex (1) defined as in (9). ATP phosphohydrolase, Mg-activated (EC 3.6.1.4.).

purification procedure was similar to (8), except for the Triton concentration which was increased for efficient enzyme extraction. Also, a higher glycerol concentration had to be used in the gradient, probably due to the lower lipid content in our preparation. ATPase activity was measured at 30° by coupling the pH change caused by ATP hydrolysis with reduction of phenol red (spectrophotometrically recorded at 559 nm). Further details in the legend of figure 1. ATP hydrolysis was also measured by inorganic phosphate liberation (9). Proteins were measured on whole cells as in (10) or on subcellular and purified ATPase fractions as in (11).

RESULTS

The comparative study of the purified ATPase complexes from the oligomycin-sensitive wild type (O^S) and from the two oligomycin-resistant mutants by disc electrophoresis on polyacrylamide-SDS gels (12) resulted in the characteristic 8 bands described for commercial baker's yeast (8). When quantities above 150 µg protein were analyzed on the gels, 3 or 4 additional minor bands were disclosed, representing a maximum of 10 % impurities. The same subunits were present in the three cases, without significant differences in band intensity : the genetically determined modification may consist of only a small change of one of the subunits, undetectable by this technique.

The thermal inactivation profiles of the ATPase complexes from the three strains were qualitatively similar in the temperature range examined (35 to 56°, fig. 1). The same thermal treatment, carried out in the presence of ADP or ATP, showed a completely different profile (fig. 2) : while the ATPase activity from the O^S strain was only protected by ADP, it was strongly activated in the case of the resistant mutant strains. Fig. 3 shows the concentration dependence of the ADP effect : in all cases, the threshold was situated around 40 µM, the half-maximal effect being between 200 and 300µ M. ATP produced protection, respectively activation, at similar concentrations. Inorganic phosphate (4 mM) produced neither protection, nor activation during heat treatment. (Heat treatment, with or without ADP, produced complete disappearance of oligomycin-sensitivity, in accordance with (13).

The activation produced during heat treatment in the presence of ADP or ATP could be due to various causes, such as structural changes in the ATPase complex, detachment of an inhibitor, perturbation of lipids, or simply recovery of activity specifically lost by the ATPase from the mutants at a purification step such as Triton treatment. This last hypothesis was tested in the first place because a) differential sensitivity to Triton X-100 of the mitochondrially integrated ATPase from the mutant strains was observed (9), b) ADP-dependent heat activation could be produced not only with the

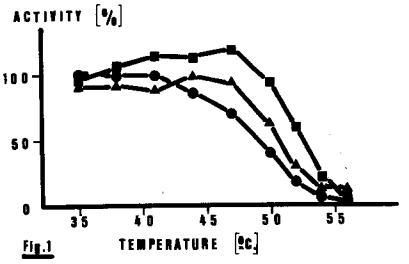

Fig.1

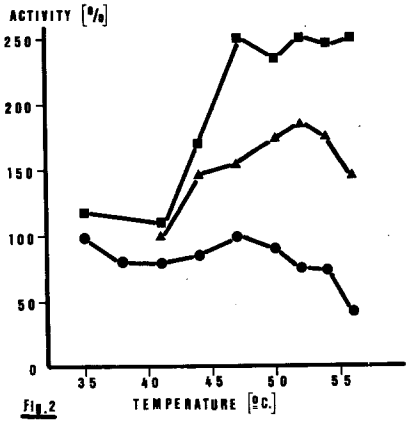

Fig.2

Figure 1 : Activity of the ATPase complex tested at 30° after preincubation at different temperatures in the absence of ADP. ATPase (100 µl in Tris-acetate 5 mM, pH 7.5, glycerol 20 %, Triton X-100, 0.1 %) was incubated during 5 min at 35 to 56°. A 20 µl aliquot was immediately transferred to the reaction medium (ATP 4 mM, $MgSO_4$ 3.2 mM, Tris-Cl 4 mM, phenol red 0.0016 %, pH 8.4, total volume 1 ml) : this started the hydrolysis reaction followed spectrophotometrically at 559 nm. Activity of the heat-treated enzyme was normalized for each strain, taking the activity of the untreated enzyme as reference (100 %). ●–● O^S wild type, ■–■ O^R_{144} mutant, ▲–▲ O^R_{146} mutant.

Figure 2.: Activity of the ATPase complex after preincubation at different temperatures in the presence of ADP. ATPase was incubated as in figure 1, except that 4 mM ADP was added during heat-treatment. Ordinate and code for strains as in figure 1.

purified enzyme, but already in the Triton extract (scheme 1) from the mutant strains.

Table I shows the balance sheet of the Triton extraction of the ATPase: the addition of Triton to the mitochondrial pellet produced a significant decrease in ATPase activity in the Triton supernatant. Therefore the balance without heat treatment was more unfavourable for the mutant than for the wild type (line e). However, heating the Triton supernatant in the presence of ATP produced a nearly twofold increase of ATPase activity in the case of

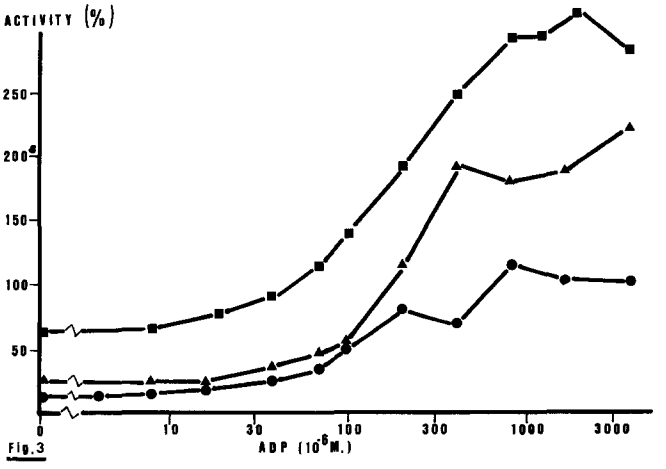

Figure 3 : Activity of the ATPase complex after heat-treatment in the presence of different ADP concentrations. Experimental details as in figure 1, except that preincubation was at 52° (5 min), in the presence of different ADP concentrations. Activity was again tested at 30°. Ordinate and code for strains as in fig. 1.

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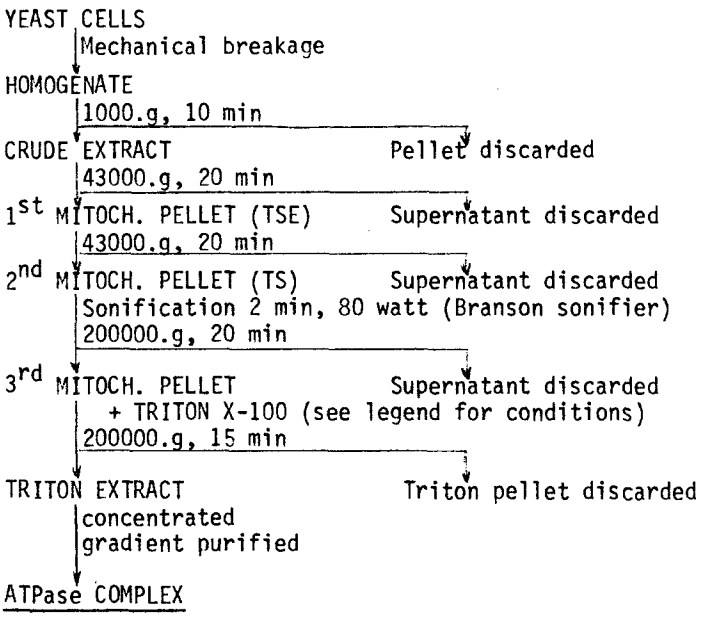
the mutant, but only a slight increase for the wild type (lines f and d). As a consequence, the balance now became equal for both strains (line g).

The calculation at the end of Table I shows that the increase of ATPase activity produced by heat treatment is compatible with a reactivation rather than an activation process, as the recovered activity is not superior to that lost by Triton addition. In fact the statistical mean degree of reactivation calculated for the mutant at locus O_{II} from 19 independent determinations (155 % $\pm$ 31) is not significantly different from the maximum calculated in Table I (123 %). The ATPase from the wild type, only slightly inactivated by Triton, was only slightly reactivated by the heat treatment : - 5% $\pm$ 7 (17 independent determinations). The enzyme from the mutant at locus O_I also appeared to be prone to reactivation but to a smaller degree than the enzyme from the mutant at locus O_{II} : 69 % $\pm$ 19 (11 independent determinations)

We are dealing with mutations of the mitochondrial DNA presumably resulting in a change of the "membrane factor" of the ATPase complex. Therefore it was essential to verify that the above described heat reactivation of the ATPase from the oligomycin resistant mutants did not occur when the enzyme was devoid of some of the subunits of the "membrane factor". We have used two different methods :

1) the catalytic part F_1 (2) without the "membrane factor" of the ATPase complex was prepared by a method (14) which does not use detergents. In the case of the mutant (locus O_{II}), heat treatment (45 to 52°) applied to

SCHEME 1 : Preparation of Yeast ATPase Complex sensitive to oligomycin.



All operations were carried out at ice temperature. Buffer TS = TSE minus EDTA. Extraction by Triton : 1 vol. of mitochondria (around 20 mg protein/ml in Tris/acetate 5 mM, pH 7.5) + 0.2 vol. of Triton X-100 (Packard) 10 % w/v, final ratio : 1 mg Triton/mg protein. 2 ml of the 6-8 fold concentrated Triton extract (Centriflo Cones CF 20 Amicon) were layered on top of a linear gradient (10 to 25 % glycerol, Tris-acetate 5 mM, Triton X-100 0.1 %, pH 7.5, volume 30 ml). After centrifugation in a SW-25 rotor during 15 h at 25000 rpm, 15 fractions were collected. The 3 fractions of best specific activity were pooled (= ATPase complex). Specific activity about 140 µmoles P_i/mg protein/ 10 min, tested at 30°.

F_1, pretreated or not with Triton 0.1 %, produced inactivation, and ADP gave neither protection nor reactivation of enzyme activity.

2) The mutant at locus O_{II} and the O^S wild type were grown in the presence of 2 mg/ml chloramphenicol. In these conditions, presumably due to the non functioning of the mitoribosomes, the oligomycin-resistance of the mitochondrial ATPase is complete (15), instead of partial as in the case of the mutant grown in normal conditions (6). Employing the usual method of preparation for the ATPase complex from normally grown cells, including Triton-extraction (8), we obtained an ATPase which was completely resistant to 13 µg/ml oligomycin. A balance sheet similar to that of Table I showed that Triton decreased the ATPase activities from the O^S and O^R_{144} strains

§ The apparently greater heat resistance of the mutant enzyme (fiG. 1) could be the result of these two opposing effects.

TABLE I : Balance sheet of Triton-extraction of mitochondrial ATPase with or without heat-treatment (wild type and mutant) : activities were measured by P_i liberation following ATP hydrolysis.

	TOTAL ACTIVITY		ACTIVITY REFERRED TO MITOCHONDRIA (%)	
	O^S	O^R_{144}	O^S	O^R_{144}
a) MITOCHONDRIA (2nd mitoch. pellet in Scheme 1)	1458	1255	100	100
b) MITOCHONDRIA + TRITON	1250	746	86	59
c) TRITON PELLET	385	344	26	27
d) TRITON SUPERNATANT	724	413	50	33
e) TRITON PELLET + SUPERNATANT (Balance without heat-treatment)	1109	757	76	60
f) HEAT- and ATP-TREATED SUPERNATANT	832	729	57	58
g) PELLET + HEAT- and ATP-TREATED SUPERNATANT (Balance after heat-traetment)	1217	1073	83	85
h) ACTIVITY LOST by Triton-treatment of mitochondria (line a minus line b)	208	509		

	O^S	O^R_{144}
DEGREE OF REACTIVATION :		
- experimentally obtained (f - d)/d	15 %	76 %
- predicted for total recovery (h/d)	29 %	123 %

(chloramphenicol-grown) to the same extent : respectively 23 and 21 %. This lost activity could not be recovered by heat- and ADP-treatment.

DISCUSSION

We have shown that the mutational modification of a product of two mitochondrial genes has conferred a particularly high Triton-sensitivity to the ATPase complex (see also (9)) : in the presence of Triton, ATPase activity of the wild type is practically unchanged, while it is strongly decreased for the mutant. The heat treatment presumably alters the interaction between detergent and protein, resulting in reactivation of the mutant enzyme ; on the other hand, the heat treatment also denatures the enzyme, unless adenine nucleotides are present . Under the influence of two other independent mutations at locus O_I (O^R_4 and O^R_7), the ATPase complex was reactivated by heating in the presence of ADP.

The half-maximal ADP concentration for heat-reactivation was found to be very similar to that for heat-protection (about 200 µM) ; this suggests

that the fundamental difference between the mutant and wild type ATPase complex does not concern the site of adenine nucleotide fixation itself. The main difference consists in the fact that in the presence of Triton the activity of the mutant enzyme is decreased much more than that of the wild type. Therefore it is the hydrophobic part of the ATPase complex interacting with the detergent which appears to be modified by the mutation. The implication of subunits synthesized on mitoribosomes is shown by the fact that the ATPase from the mutant, devoid of its mitochondrially synthesized subunits (ATPase from chloramphenicol-grown yeast) no longer differed in Triton-sensitivity from the wild type.

It is well known that lipids accompany the ATPase complex to the last step of purification (8). It is well known also that oligomycin-sensitivity requires lipids (16). The relative degree of oligomycin-resistance for the mutant strains compared to each other and to the wild type was the same for the purified ATPase complexes and the mitochondrially integrated ATPase (6). It is tempting to speculate on the possible relationship between oligomycin-resistance and Triton-sensitivity of the mutant ATPase. Such a relationship is suggested by the fact that Triton produces a considerable increase in oligomycin-sensitivity of yeast mitochondrial ATPase (9). Further studies are necessary to determine among the components of the ATPase complex, the ones which are genetically determined by mitochondrial DNA.

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