ATPASE FROM MITOCHONDRIALLY DETERMINED OLIGOMYCIN-RESISTANT MUTANTS OF *S. CEREVISIAE* : EFFECT OF TRITON X-100 AND PHOSPHOLIPASE A ON OLIGOMYCIN-SENSITIVITY OF ATPASE FROM MITOCHONDRIA AND PROMITOCHONDRIA.

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SUMMARY : Modification of the phospholipid environment of yeast mitochondrial ATPase by Triton X-100 or Phospholipase A reveals considerable latent oligomycin-sensitivity from fully developed mitochondria, while the oligomycin-sensitivity from promitochondrial ATPase (anaerobic yeast) was increased to a much slighter extent. Genetically determined differences in oligomycin-sensitivity (mutations at the mitochondrial loci O_I and O_{II}) subsist even in this modified phospholipid context.

Oligomycin-sensitivity of yeast mitochondrial ATPase has recently been used as a quantitative parameter (1) to characterize changes in mitochondrial differentiation : these changes were produced either by differences in physiological development, or by modification of the mitochondrial genome. In the first case, it was concluded from the study of different stages of mitochondrial biogenesis that the oligomycin-sensitivity of ATPase from fully developed mitochondria (aerobically grown yeast) was identical to that of ATPase from promitochondria (anaerobically grown yeast) (1). In the second case, it was shown that two unlinked mutations of the mitochondrial genome (2), carried by oligomycin-resistant yeast strains, conferred distinctly different, lowered oligomycin-sensitivities to the respective mitochondrial and promitochondrial ATPases (loci O_I and O_{II}) (1).

Phospholipids are an integral part of the mitochondrial membrane. In the particular case of the ATPase complex[§], phospholipids are associated with a hydrophobic constituent, called the "membrane factor", part of which, following the criterium of chloramphenicol-inhibition, is synthesized on mitoribosomes (3). Oligomycin-sensitivity requires not only OSCP (4) but also phospholipids (5) and the "membrane factor". Based on these considerations, we have examined the genetically or physiologically determined oligomycin-sensitivity in a new context created by modifying the phospholipid environment of the mitochondrially integrated ATPase.

MATERIALS AND METHODS

Chemicals : Phospholipase A, Crotalus terr.terr.,dicyclohexylcarbodiimide =DCCD, Calbiochem. Triton X-100, Sigma Chem. C°. Other chemicals in (1). Strains (1)

§ "ATPase complex" (3) stands for F_1 (6) associated with the "oligomycin-sensitivity conferring protein" OSCP (4) and the "membrane factor" (+ phospholipids). ATP phosphohydrolase, Mg-activated (EC 3.6.1.4).

PS 194 (0^S), PS 195 (0^R_{144} mutated at locus 0_{II}), PS 211 (0^R_{146} mutated at 0_I) 0_I and 0_{II} are unlinked loci of the mitochondrial DNA (2). Cultures, subcellular fractionation, protein determination are described in (7). Cell breakage is described in (1). Yeast mitochondria and promitochondria were gradient purified (7) and the fractions of the highest ATPase activity around buoyant density 1.16 (mitochondria) and 1.24 (promitochondria) were used (7). Determination of ATPase activity : ATP 4 mM, $MgSO_4$ 3 mM, Tris-Cl (pH 8.5) 50 mM, PEP 2.5 mM, excess PEP-kinase. Total volume 1 ml. Referred to as "reaction medium". Oligomycin concentration was checked spectrophotometrically ($\varepsilon^{225}_{M\ (eth)}$ 20.000, m.w. 400). Rat liver and pig heart mitochondria were kindly supplied by Dr. B. Foucher and Dr. P. Leblanc, Institut de Biochimie de l'Université Paris-Sud, Centre d'Orsay.

RESULTS

Modifying the phospholipid environment of mitochondrial ATPase was a necessarily empirical attempt : it was impossible to predict how a given agent like Triton X-100 or Phospholipase A would affect ATPase activity, and whether it would increase or decrease oligomycin-sensitivity.

Mitochondrial ATPase activity (fig. 1A) was not affected by Triton X-100 in the case of the oligomycin-sensitive wild type, while a significant inhibition consistently occurred for the two oligomycin-resistant mutants. The same qualitative difference was found for promitochondrial ATPase (fig. 1B). This characteristic Triton-sensitivity, also observed with the purified ATPase complexes from the oligomycin-resistant mutants, was responsible for the spectacular thermal reactivation described in the accompanying paper (8).

For a given Triton concentration, and with varying concentrations of mitochondria and promitochondria, a) the degree of ATPase inhibition (fig. 2) and b) the degree of oligomycin-sensitivity (fig. 4) were constant. This indicated that the Triton concentration, rather than the Triton/protein ratio, might be the experimentally significant parameter in our conditions. Consequently all our graphs indicate the added Triton in µg/ml, even when comparing mitochondria and promitochondria (µg Triton/mg protein can be readily calculated).

Oligomycin-sensitivity of mitochondrial ATPase was strongly stimulated by increasing Triton concentrations (fig. 3A). The same observation was made to a slighter extent, for promitochondrial ATPase (fig. 3B). With 0.5 µg/ml oligomycin, Triton saturation was attained at 50 and 100 µg/ml, respectively, for mitochondria and promitochondria. Using this saturating Triton concentration, and the same constant oligomycin concentration, the effect of Triton on oligomycin-sensitivity was examined with varying concentrations of mitochondria and promitochondria (fig. 4A-B).

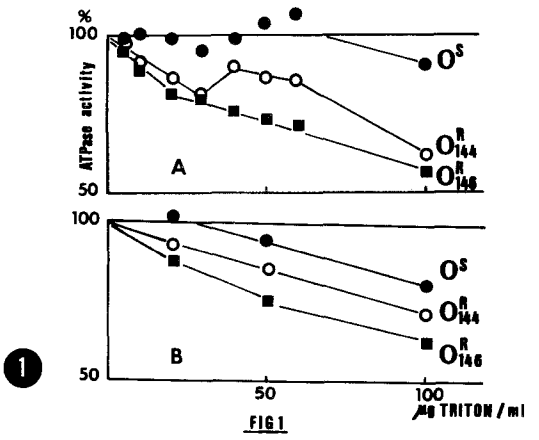

FIG 1

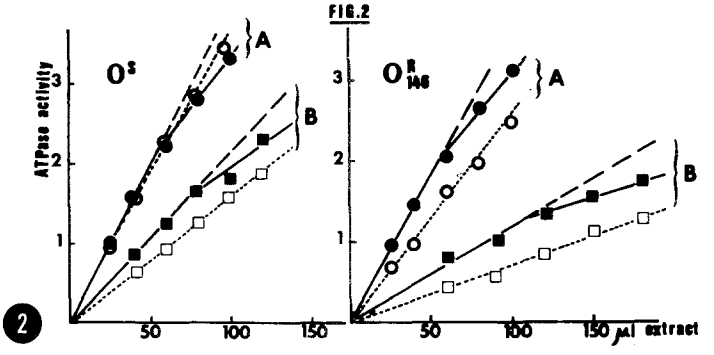

Figure 1: Effect of Triton X-100 on ATPase activity : increasing Triton concentrations. Each point is a mean of 3 to 12 determinations. A: mitochondria 150 µg, B: promitochondria 700 µg protein were incubated in the complete reaction medium, with or without Triton, 10 min at 30°. The reaction was started by ATP. Specific activity : mitochondrial ATPase 30, promitochondrial ATPase 3 µmoles P_i/10 min/mg protein (30°).

Figure 2: Effect of Triton on ATPase activity : increasing enzyme concentrations. ATPase activity in µmoles P_i/10 min/ml incubate. Full signs : no Triton. Empty signs : + Triton (50 µg/ml for mitochondria, 100 µg/ml for promitochondria). Protein concentrations varied from 50 to 250 µg/ml (A:mitochondria) and from 200 to 800 µg/ml (B: promitochondria). Details see fig. 1.

Finally the effect of a saturating Triton concentration was studied with varying lower oligomycin concentrations (fig. 5). It was shown previously that the degree of oligomycin-inhibition of yeast mitochondrial and promitochondrial ATPase was independent of unrelated proteins, i.e. the significant experimental parameter was the oligomycin concentration rather than its ratio to protein (1).

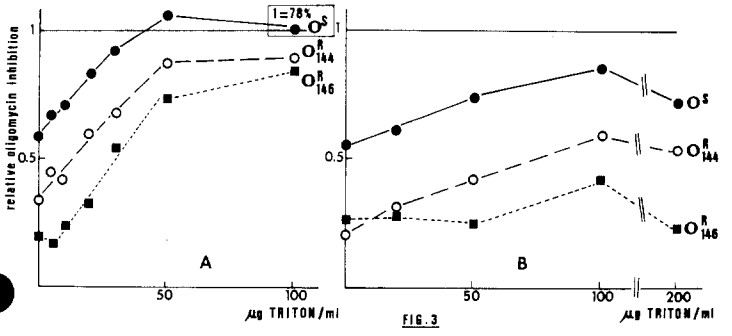

Figure 3: Effect of Triton on oligomycin-sensitivity : increasing Triton concentrations. Constant oligomycin concentration : 0.5 µg/ml. Ordinate : relative oligomycin inhibition : 1 = 78% = oligomycin-inhibition of mitochondrial ATPase with 100 µg/ml Triton (O^S). A: mitochondria 100 µg/ml; B: promitochondria 500 µg/ml protein, incubated 10 min. at 30° with oligomycin + Triton before starting the reaction by ATP. Oligomycin-inhibition was practically instantaneous. Controls were run in parallel.

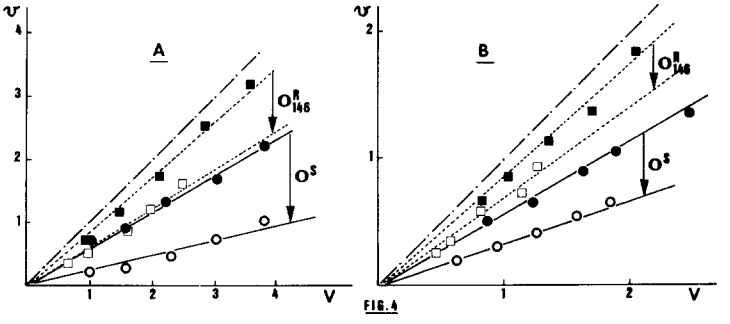

Figure 4: Effect of Triton on oligomycin-sensitivity : increasing enzyme concentrations. Constant oligomycin concentration : 0.5 µg/ml. Constant Triton concentration : A: 50 µg/ml (mitochondria), B: 100 µg/ml (promitochondria). Abscissae : V = ATPase activity (legend fig. 2) without oligomycin. Ordinates: v = ATPase activity + oligomycin. Increasing enzyme amounts (legend fig. 2) were incubated as in fig. 3. Arrows indicate effect of Triton on oligomycin-sensitivity. Bisector : "no inhibition".

In order to study the effect of Phospholipase A, the experimental conditions (Ca^{++} concentration, length of incubation, Phospholipase concentration) were chosen to yield a maximal effect on oligomycin-sensitivity, which was stimulated for all three strains (fig. 6). The apparent affinity for lower oligomycin concentrations was increased less by Phospholipase than by Triton X-100 (results not shown).

DISCUSSION.

We have shown that the apparent affinity of yeast mitochondrial ATPase

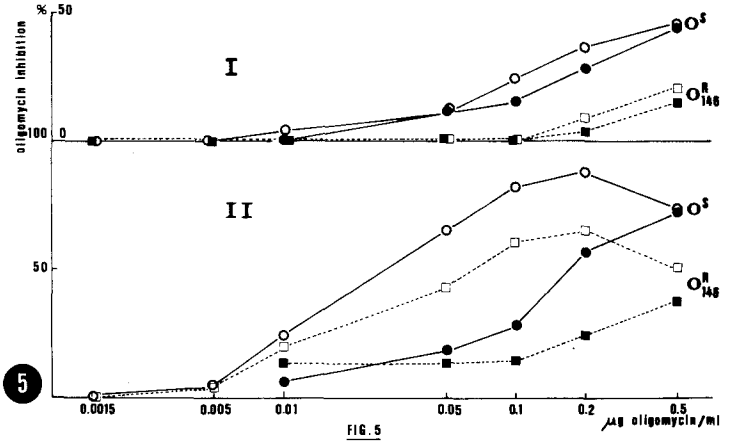

FIG. 5

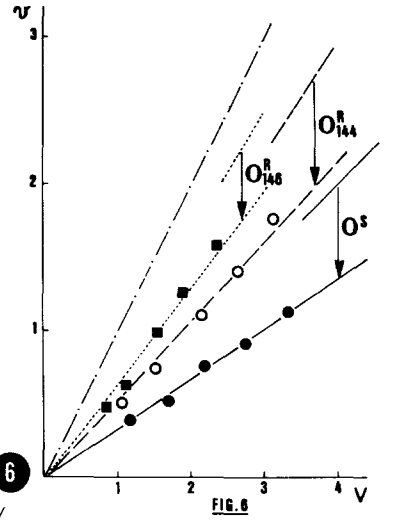

FIG. 6

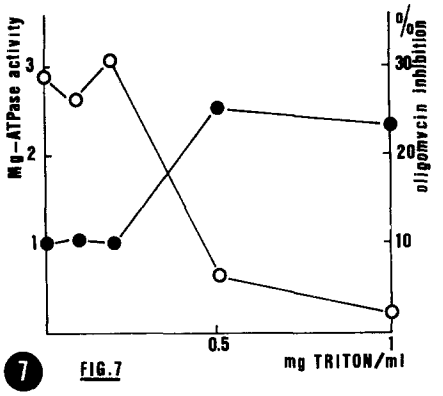

FIG. 7

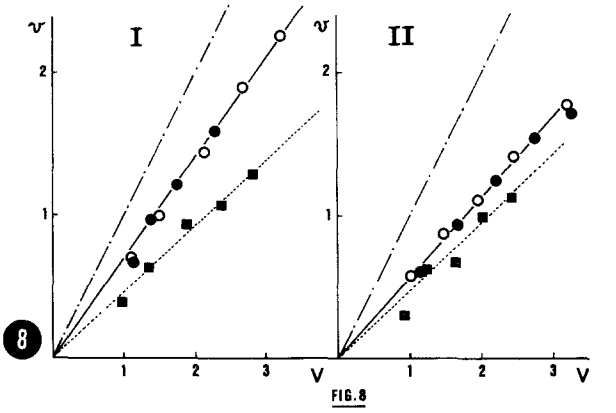

FIG. 8

Figure 5 : Effect of Triton on oligomycin-sensitivity : increasing oligomycin-concentrations. Constant Triton concentration : 100 µg/ml. Full signs : promitochondria (500 µg/ml protein), empty signs : mitochondria (100 µg/ml). Incubation as in fig. 3 ; I : no Triton. II : + Triton.

Figure 6 : Effect of Phospholipase A on oligomycin-sensitivity : increasing enzyme concentrations. Constant oligomycin concentration : 0.5 µg/ml. Phospholipase A 2 µg/ml. Mitochondria (100 µg/ml protein) incubated 1 hour at 37° with $CaCl_2$ 3 mM in the usual reaction medium, with or without Phospholipase A. After transfer to 30°, oligomycin was added and incubation continued for 5 min. The reaction was started by ATP. ATPase activity of controls was 25 % inhibited by Ca^{++} (present besides Mg^{++}). Abscissae and ordinates see fig. 4. Arrows show effect of Phospholipase A on oligomycin-sensitivity (complete control lines not shown for lack of space). Bisector : "no inhibition".

Figure 7: Effect of Triton on oligomycin-sensitivity of pig heart mitochondria: increasing Triton concentrations. Constant oligomycin concentration : 0.2 µg/ml. 800 µg mitochondrial protein incubated with Tris-Cl 50 mM (pH 7.5) + Mg^{++}, PEP and PEP-kinase (Methods). Similar results were found at pH 8.5, ± 0.25 M sucrose. ATPase activity : ● — ● Oligomycin-sensitivity : ○ — ○

Figure 8: Effect of Phospholipase A on DCCD-sensitivity : increasing enzyme concentrations. Constant DCCD concentration : 5.10-7 M. The aqueous solution was kept only 5 min (ethanol stock solution). Mitochondria incubated with or without Phospholipase A as in fig. 6, then with DCCD 5 min at 30°. Reaction started by ATP. Abscissa: V = ATPase activity without inhibitor. Ordinate : v = ATPase activity with DCCD. I: no Phospholipase A. II: preincubated with Phospholipase A. ● O^S, ○ O^R_{144}, ■ O^R_{146}

for oligomycin can be strongly increased by Triton X-100 (fig. 5) at concentrations which are considerably lower than the critical micelle concentration (cmc 0.24 mM). The half-effect on the stimulation of oligomycin-sensitivity was obtained with 0.03 mM Triton which, interestingly enough, is quite similar to its half-effect on the inhibition of mitochondrial respiratory control (0.05 mM (9)).

At Triton concentrations inferior to the cmc, monomers of the detergent might be incorporated into the membrane (cf.(10)) and thereby facilitate the access of oligomycin to its reactive site on the ATPase complex. The lysophosphatids produced by Phospholipase A might produce an increase of oligomycin-sensitivity in a similar way. Access of oligomycin through a phospholipid barrier thus seems to be the limiting factor of oligomycin-sensitivity for yeast mitochondrial ATPase. For 50 % inhibition (O^S), instead of 0.5 µg/ml oligomycin (or 3 µg/mg protein), only 0.03 µg/ml (or 0.2 µg/mg) were needed in the presence of saturating Triton. This increased degree of oligomycin-sensitivity, even when expressed as µg/mg protein, is quite similar to that of Mammalian mitochondrial ATPase. In an attempt to verify whether oligomycin-sensitivity of Mammalian mitochondrial ATPase can be further stimulated by detergent, we treated pig heart mitochondria with increasing Triton concentrations (fig. 7). At the same Triton concentrations, oligomycin-sensitivity of pig heart mitochondrial Mg-ATPase was unchanged ; at higher concentrations it was suppressed, while ATPase activity was increased.

As regards the comparison of yeast mitochondrial and promitochondrial ATPase (fig. 3-4-5) : in the absence of Triton (fig. 5-I), promitochondrial and mitochondrial oligomycin-sensitivity was very similar (an extension of our earlier findings with higher oligomycin concentrations (1) ; see also fig. 3-4 with 0.5 µg/ml). However, in the presence of saturating Triton, a much higher oligomycin-sensitivity was revealed with mitochondrial than with promitochondrial ATPase (fig. 5-II). This indicated that in the case of promitochondrial ATPase, either the phospholipid barrier to the access of oligomycin is not detergent-sensitive, or the access of oligomycin is not the limiting factor for oligomycin-sensitivity. In the last case some protein part of the "membrane factor" might be less differentiated in promitochondria than in fully developed mitochondria.

Finally, we have shown that not only the oligomycin-sensitivity of the ATPase from the wild type, but also from the oligomycin-resistant mutants could be strongly stimulated by Triton or Phospholipase A (fig. 3 to 6). However, even in the presence of these agents, a clear difference in oligomycin-sensitivity subsisted between the wild type and the mutants. The most quantitative representation of this fact is given by figure 4 and 6.

In contrast, another difference between the ATPase from the three strains did not subsist after Phospholipase treatment : the comparison of DCCD-sensitivity showed, interestingly enough, that the ATPase from the mutant O^R_{146} (the most highly oligomycin-resistant) was distinctly more sensitive to DCCD than the wild type or the mutant O^R_{144} which could not be distinguished from each other (fig. 8-I). However, after incubation with Phospholipase A, DCCD-sensitivity of the ATPase from the two latter strains was stimulated so that the three strains became very similar (fig. 8-II).

In conclusion, yeast mitochondrial but not promitochondrial ATPase contains considerable latent oligomycin-sensitivity which can be revealed by modification of the phospholipid environment. On the other hand, the genetically determined differences in oligomycin-sensitivity subsist even in this modified phospholipid context.

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