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Effects of oligomycins on adenosine triphosphatase activity of mitochondria isolated from the yeasts *Saccharomyces cerevisiae* and *Schwanniomyces castellii*

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Abstract

Functional mitochondria with respiratory control were isolated from the yeasts *Saccharomyces cerevisiae* and *Schwanniomyces castellii*. The presence of site I in *Schw. castellii* was indicated by higher ADP/O ratio than in *S. cerevisiae* where this site is absent. The ATPase V_{max} was higher in *S. cerevisiae* than in *Schw. castellii* mitochondria. The latter was increased by the DR12 nuclear mutation. Nevertheless, the stimulation by heat and the inhibition profile of oligomycins on mitochondrial F1–F0 ATPase activities were similar in all three tested strains. In *S. cerevisiae* and *Schw. castelli* wild type or mutant mitochondria, the well-known inhibition of F1–F0 ATPase activity by low concentrations of oligomycins is abolished at high inhibitor concentrations near 60 μg/ml suggesting uncoupling of F1 activity. At still higher oligomycin concentration the ATPase activity of both species and mutant is again strongly inhibited, suggesting an inhibitory effect on yeast F1 activity not detected so far.

Keywords: Mitochondrial ATPase; Respiratory control ratio; Oligomycins; *Saccharomyces cerevisiae*; *Schwanniomyces castellii*

The yeast mitochondrial ATP synthase F1–F0 complex is not very different from that of other organisms [1–3]. For many reasons, yeasts are good models for the study of the structure and function of mitochondrial enzymes [4–6]. The availability of yeast nuclear and mitochondrial genome sequences [7,8], the possibility to modulate the aerobic and anaerobic metabolism, and the availability of classical and molecular genetics provide unique tools in this respect [9–12].

The central role of the mitochondrial ATPase in coupling of respiratory chain with oxidative phosphorylation is well known but other physiological functions such as cell apoptosis and aging [13] or maintenance in mitochondrial DNA [5] have recently been identified. However, little is known about the regulation of ATPase biogenesis and its role in regulatory pathways or energy transduction [14]. Inhibition of oxidative phosphorylation by oligomycins is a useful tool to study the role of ATPase/synthase in physiological functions of eucaryotic cells [1,10,11]. Oligomycins introduced as an inhibitor of oxidative phosphorylation [10] inhibit only when the F1 ATPase is bound to the F0 complex in mitochondrial membranes and retains a specified protein (subunit 7) named by Tzagoloff "OSCP" (oligomycin-sensitive-conferring protein). Therefore, oligomycins do not affect the activity of the purified F1 ATPase [2,11]. Moreover, oligomycins inhibit the plasma membrane Na–K ATPase and yeast multidrug-ATPases as well [11,12].

We explored the effect of oligomycins and other effectors of ATPase activity such as heat and pH on two yeast species of very different mitochondrial activity. Even though the phosphorylation site I typical for higher eukaryotes is present in *Schwanniomyces castellii*

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[15,16], whereas it is absent in *Saccharomyces cerevisiae* [9], the ATPase activity measured in intact mitochondria is quantitatively but not qualitatively affected by the respiratory mutation DR12. In particular, we show that a novel triphasic mode of inhibition of the mitochondrial ATPase activity by increasing concentrations of oligomycins is observed in both yeast species.

Materials and methods

Yeast strains and growth conditions. *Saccharomyces cerevisiae* KM91 is the wild diploid prototrophic laboratory strain [17]. *Schw. castellii*[2] CBS2863 is the wild haploid parental strain from which the respiratory deficient mutant DR12 has been isolated [15,16]. The complete glucose YP (0.5) medium contains: 0.5% yeast extract (Difco), 1% bactopeptone (Difco), 0.5% glucose, 0.08 ml/liter $(NH_4)_2SO_4$, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $CaCl_2$, and 0.0008% $FeCl_3$ ($D = 1.27$ g/ml). The complete glucose YP (0.3) medium has the same composition but contains only 0.3% glucose. Cultures were inoculated at 10^6 cells/ml, from an actively growing preculture, and grown aerobically with shaking for 22 h in 500 ml YP (0.5) medium and then for 2 h more in the medium plus 750 ml YP (0.3).

Isolation of mitochondria and determination of mitochondrial respiratory control rate. Mitochondria were prepared as previously described by McKee and Poyton [19] and Zagorski et al. [20]. The final pellet was suspended in the medium containing 0.6 M sorbitol (Sigma) and 0.5% BSA (Sigma) and frozen in liquid nitrogen −195 °C. Respiration rates were measured polarographically at 28 °C using a Clark-type oxygen electrode (Rand Rank Brothers Fisher Bioblock Scientific Polystat 33) equipped with a standard Teflon membrane. The reaction was started by the introduction of the mitochondria into the reaction chamber containing 2.0 ml of air-saturated 0.6 M sorbitol with K_2HPO_4/KH_2PO_4 10 mM, pH 7.4, plus α-ketoglutarate 50 mM, NADH 1 mM or ADP 15–100 μM when necessary [21]. Rotenone was used at the following final concentration: 0.1 mM. The mitochondrial protein concentration in the reaction chamber was 0.15–0.3 mg/2 ml.

ATPase assays. ATPase assays were performed at 37 °C for 16 min, in a total volume of 1.0 ml reaction mixtures containing: 7 mM ATP, 7 mM $MgSO_4$, Tris 58.9 mM, and sodium orthovanadate 10 μM (Sigma) to eliminate some residual contamination of plasma membrane ATPase activity and different concentrations of oligomycins ABC (Sigma) as described in Results. Oligomycins A and B applied separately have approximately equal power in inhibiting ATPase activity. The pH of reactions mixture was brought to 9.0 or 10.0 with NaOH. The reaction was stopped by addition of 2 volumes of 7% (w/v) SDS (Sigma). Inorganic phosphate was measured as described by Pullman et al. [22]. The reaction was followed photometrically with a Klett–Summerson colorimeter equipped with a red filter. Protein contents were determined by the method of Smith et al. [18] using bicinchoninic acid with bovine serum albumin as a standard.

[2] The wild type Schwanniomyces we used is listed in the Centraalbureau voor Schimmelcultures (CBS) collection under the number 2863, which corresponds to the *Schw. castellii* Capriotti type strain in the previous classification. Despite several differences in utilization of carbon substrates it was later on proposed to regroup it with *Schw. occidentalis*. More recently the high degree of identity of the ribosomal RNA sequences suggested the transfer of *Schwanniomyces occidentalis* to the genus *Debaryomyces*. However, this denomination is not unanimously accepted. We used here the previous nomenclature found in most publications concerning the yeast Schwanniomyces CBS 2863 and its DR12 mutant.

Results

The respiratory functions of mitochondria isolated from Saccharomyces cerevisiae and Schwanniomyces castellii cells

Mitochondria were isolated from *S. cerevisiae* wild type strain KM91 [23], from *Schw. castellii* CBS2863 and its respiratory deficient mutant DR12 [15,16,24]. As shown in Table 1 all mitochondria at pH 7.4 exhibited respiratory control with α-ketoglutarate as substrate and exhibited a good ADP/O ratio even after several months of preservation in liquid nitrogen [20]. Higher ADP/O and respiratory control values in the case of mitochondria isolated from the wild type *Schw. castellii* CBS2863 have been observed. At pH 9.0 or treatment at 37 °C for 45 min the respiratory control and coupling with oxidative phosphorylation were lost. Sensitivity to 0.1 mM rotenone confirmed the existence of a functional site I of phosphorylation in *Schw. castellii* (data not shown). The *Schw. castellii* mutant DR12 showed decreased respiratory control and ADP/O ratio.

Influence of pH and heat on the mitochondrial ATPase activity of Saccharomyces cerevisiae and Shwanniomyces castellii

Dixon plot indicates a higher affinity of *S. cerevisiae* KM91 enzyme for MgATP ($K_m = 0.5$) than that of *Schw. castellii* CBS2863 wild type strain ($K_m = 2.0$). The mutation of DR12 leading to a partial respiratory deficient phenotype also results in higher affinity ($K_m = 1.5$) of the enzyme for ATP. The K_m values of mitochondrial ATPase of both yeast species were not modified by 1 h incubation at 37 °C instead of 28 °C (data not shown).

Table 1
Oxidative phosphorylation index of isolated mitochondria from both yeast species

Strain	pH	Value (mean ± SE)*	
		RC	ADP/O
Schw. castellii			
CBS 2863	7.4	2.39 ± 0.10 (3)	1.60 ± 0.48 (3)
	9.0	1.00 ± 0.00 (3)	0.00 ± 0.00 (3)
Schw. castellii			
DR12	7.4	1.72 ± 0.19 (3)	0.89 ± 0.12 (3)
	9.0	1.06 ± 0.60 (3)	0.44 ± 0.44 (3)
S. cerevisiae			
KM91	7.4	1.52 ± 0.21 (3)	1.00 ± 0.40 (3)
	9.0	1.10 ± 0.30 (3)	0.98 ± 0.14 (3)

The ADP/O and respiratory control ratios (RC) were measured polarographically at 28 °C either at pH 7.4 or 9.0 using α-ketoglutarate as substrate as described under "Materials and methods." In the reaction chamber 200 μg of mitochondrial protein was used.

* Values in parentheses indicate the number of mitochondrial preparations analyzed.

Table 2
Kinetic properties of mitochondrial ATPase of *S. cerevisiae* and *Schw. castellii*

	S. cerevisiae KM91		*Schw. castellii* CBS2863		*Schw. castellii* DR12	
	28 °C (SE*)	37 °C (SE*)	28 °C (SE*)	37 °C (SE*)	28 °C (SE*)	37 °C (SE*)
ATPase V_{max} (μmol Pi min^{-1} mg^{-1})						
pH 9.0	3.02 ± 0.27	4.51 ± 0.53	0.40 ± 0.17	1.29 ± 0.21	1.01 ± 0.26	2.63 ± 0.25
pH 10.0	3.43 ± 1.37	6.33 ± 0.65	1.67 ± 0.09	3.33 ± 0.55	2.35 ± 0.47	5.24 ± 0.76
K_m (mM)		0.5–1.0		2.0		1.5
K_i for ATPase (μg oligomycin/ml)	0.25	0.40	0.50	0.55	0.50	0.50

ATPase assays (see Materials and methods) were carried out either at 28 or at 37 °C (pH 9.0 and 10.0, other intermediate data points at pH 8.5, 9.5, 10.5, and 11.0 are not shown). K_m by presentation of Dixon (see Fig. 2) plots was estimated at pH 10.0 (37 °C). K_i was also determined by Dixon plots (see Fig. 2) in the presence of different concentrations of oligomycins from 0 to 2.0 μg/ml at pH 9.0.

* The data represent means ± SE of three to six different experiments.

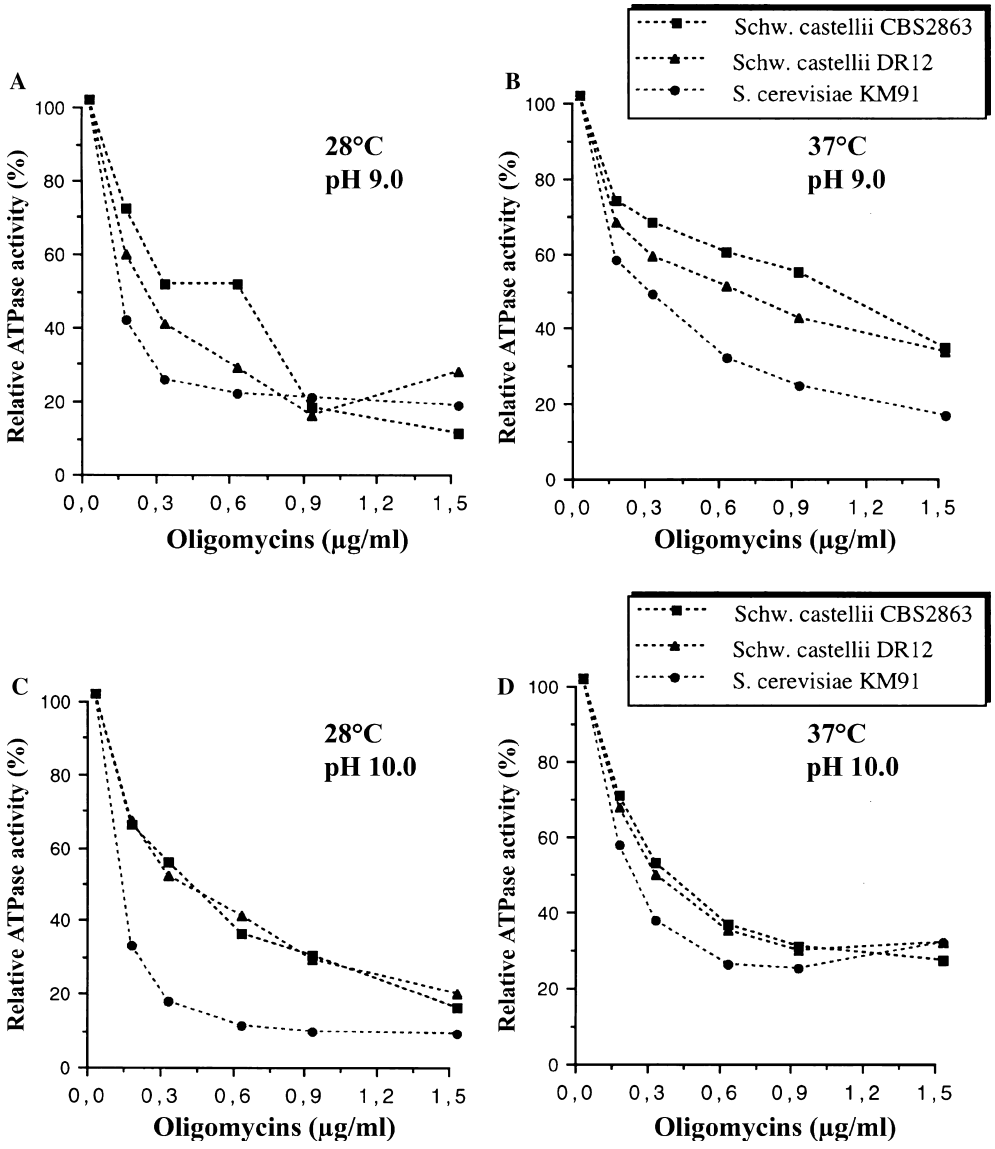

Fig. 1. Oligomycin-inhibition curves of mitochondrial ATPase activity at pH 9.0 and 10.0 of *S. cerevisiae* and *Schw. castellii*. The assay conditions were as described in Materials and methods. The reaction was carried out at 28 °C or at 37 °C at pH 9.0 (A,B) or pH 10.0 (C,D) in the presence of different concentrations of oligomycin (from 0 to 1.5 μg/ml) and ATP (7 mM) as a substrate.

Inhibition and activation by oligomycins

The optimum pH for ATPase activity of *S. cerevisiae* has been reported to be around 9.0 [14]. However, Table 2 shows that for both *S. cerevisiae* and *Schw. castellii* CBS2863 and DR12 higher activity is obtained at pH 10.0. In all conditions the ATPase activity is markedly stimulated by incubation at 37 °C during 16 min. Table 2 shows that the ATPase activity is much lower at pH 9.0 (28 °C) for both *Schw. castellii* strains (0.40 or 1.01 μmol Pi min^{-1} mg^{-1}) than that of the *S. cerevisiae* (3.02 μmol Pi min^{-1} mg^{-1}). The higher ATPase activity of *S. cerevisiae* DR12 mitochondria in comparison with its wild type parental strain is associated to the mutation DR12 leading to partial respiratory deficiency [16,24]. Both strains of *Schw. castellii* exhibited much higher specific activity than *Schw. castellii* ATPase at pH 10.0 both at 28 °C (1.67 and 2.35 μmol Pi min^{-1} mg^{-1}) and 37 °C (3.33 and 5.24 μmol Pi min^{-1} mg^{-1}). The pH 10.0 markedly affected the mitochondrial ATPase activity of *S. cerevisiae* KM91 strain but only at 37 °C (6.33 μmol Pi min^{-1} mg^{-1}).

The effect of oligomycins (ABC) on intact mitochondria was examined on heat-induced (37 °C) and non-induced (28 °C) ATPase activity. Figs. 1A and B show that a relatively high concentration of oligomycins (0.15 μg/ml) inhibits 50% of the *S. cerevisiae* KM91 ATPase activity at pH 9.0 both at 28 or 37 °C. However, at pH 10.0 (Figs. 1C and D) and 28 °C the *S. cerevisiae* enzyme is more sensitive to oligomycins. The ATPase activity of both *Schw. castellii* CBS2283 and DR12 strains is less sensitive to oligomycins with an I/50 around 0.3 μg/ml. The ATPase activity of *S. cerevisiae* strain is more resistant to this antibiotic at 37 °C both at pH 9.0 or 10.0. These results suggest that the mitochondrial ATPase activity stimulated by heat is oligomycin-insensitive.

Another difference is demonstrated for the K_i values of oligomycins. In Fig. 2 the Dixon plots show that the K_i value for the ATPase of *S. cerevisiae* KM91 strain at pH 9.0 at 28 °C was 50% lower (0.25 μM) to those of *Schw. castellii* CBS2263 and DR12 strains (0.5 μM). However, there is only a slight difference (20%) between the K_i value for ATPase of *S. cerevisiae* and *Schw. castellii* mitochondria, when tested at 37 °C (Table 2).

The Lineweaver–Burk plots of oligomycin inhibition of ATPase activity at pH 9.0 and at 37 °C in the presence of 1.5 and 60 μg/ml oligomycins indicate typical non-competitive kinetics of this inhibitor for the both yeast species enzyme (data not shown).

In the experiment of Fig. 3, at pH 9.0 and at 37 °C, significant activation of the ATPase activity (40% for *S. cerevisiae* and 70% for *Schw. castellii* mitochondria) is shown for high concentration of oligomycins (60 μg/ml). This stimulation then disappears at much higher concentrations (70–150 μg/ml) of oligomycins. This effect of oligomycins was not detected, when the ATPase activity was tested at 28 °C.

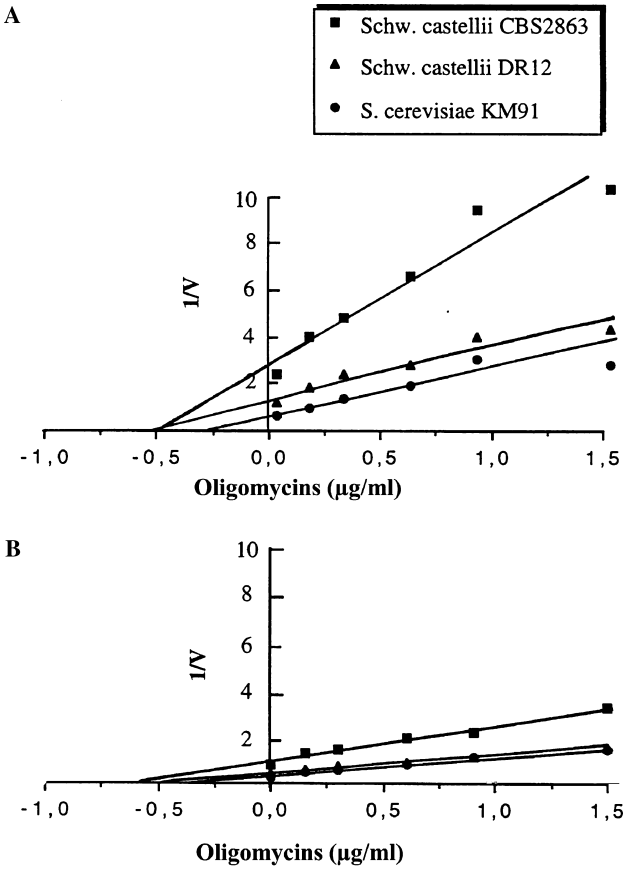

Fig. 2. Dixon plots presentation of inhibition (K_i) of mitochondrial ATPase activity in different concentrations of oligomycin. ATPase assays (see Materials and methods) were carried out at pH 9.0 either at 28 °C (A) or at 37 °C (B) in the presence of different concentrations of oligomycin (0–1.5 μg/ml). Velocity (V) of the reaction is expressed in μmol Pi min^{-1} mg^{-1} protein.

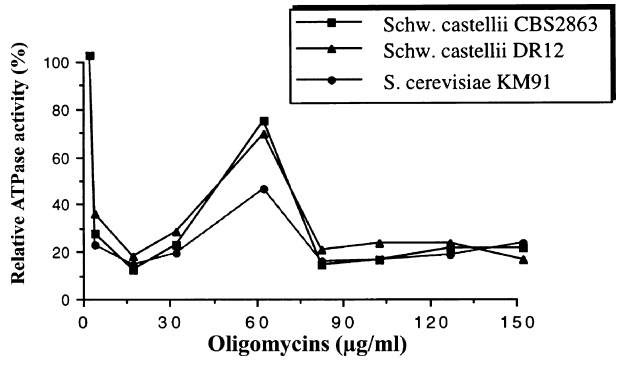

Fig. 3. Stimulatory effect of oligomycin on the total ATPase activity of mitochondria isolated from *S. cerevisiae* and *Schw. castellii*. ATPase assays (see Materials and methods) were carried out at pH 9.0 at 37 °C. The total ATPase specific activities of the untreated samples (100%) were for the strain KM91: 5.2 and for CBS2863 and DR12: 1.3 and 2.7 (μmol Pi min^{-1} mg^{-1} protein).

Discussion

We succeeded in isolating and comparing the functional properties of mitochondria of two yeast species: *S. cerevisiae* (without phosphorylation site I) and *Schw. castellii* (with site I) including the nuclear respiratory deficient mutant DR12 of the latter species. Values of respiratory control ratio and ADP/O ratio tested at pH 7.4 show the quality of isolated mitochondria stored in liquid nitrogen [4,20].

The kinetic analysis of the mitochondrial ATPase from *S. cerevisiae* and *Schw. castellii* reveals that they slightly differ in pH optimum activity, K_m for MgATP, and K_i for oligomycins. Interestingly, the mutation of *Schw. Castellii* DR12 leading to cytochrome b deficiency increases by 50% the affinity of the ATPase for MgATP compared to its wild type parental CBS2863 strain. The mechanism of this phenomenon is unknown.

The concentration of inhibitory oligomycin-binding sites in rat-liver mitochondria is 0.12 nmol/mg protein. However, at least 10 times more oligomycin can be bound to mitochondria [1]. Our data indicate that in yeast mitochondria low concentration of about 0.15 μg oligomycins corresponding to about 2 nmol/mg proteins is a powerful inhibitor of the yeast mitochondrial ATPase and is slightly more inhibitory in *S. cerevisiae* than in *Schw. castellii* preparations. The ATPase activity stimulated by heat (37 °C) is rather oligomycin-insensitive. However, the inhibition of both yeast species ATPase activity is relieved at 37 °C at oligomycin concentration around 60 μg/ml. Therefore, a net stimulation (40–70%) of ATPase activity is observed at these concentrations of oligomycins. To our knowledge such a case has not been yet reported in the literature. In the presence of inhibitory and stimulatory concentrations of oligomycins non-competitive kinetics of this inhibitor as regards MgATP has been observed. Landry and Goffeau [25] reported activations of yeast mitochondrial ATPase activity by heat (40 °C), trypsin, and high DCCD concentrations (25–100 μM) in *Schizosaccharomyces pombe*. Therefore, there is a possibility that high concentrations of oligomycin react with additional activatory binding site(s). The ATPase activity of yeast mitochondria is dramatically stimulated by uncouplers [2,25]. It might therefore be proposed that high concentrations of oligomycin may behave as an uncoupler of oxidative phosphorylation in mitochondria isolated either from *S. cerevisiae* or from *Schw. castellii*. An alternative explanation would be that oligomycin promotes the release of a natural small inhibitory protein of F0–F1 ATPase activity [13,26]. The function of this inhibitory protein called IF1 (10 kDa) would be to minimize ATP hydrolysis by F0–F1 when this complex is not operating as an ATP synthase [27]. Anyway a third inhibitory mechanism, not reported so far, seems to proceed at oligomycin concentrations higher than 60 μg/ml. As the F1–F0 complex seems uncoupled under these conditions, this inhibition may act at the F1 level independently of the species or the mutation tested.

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