Long lasting reversibility of the mitochondrial permeability transition in *Saccharomyces cerevisiae*.

Lilia Morales-García¹,²,³, Carolina Ricardez-García¹,³ Paulina Castañeda-Tamez², Natalia Chiquete-Félix³ and Salvador Uribe-Carvajal¹,*

¹ Department of Genetics and Molecular Biology, Instituto de Fisiología Celular, UNAM, Mexico City, Mexico; rcoria@ifc.unam.mx
² Department of Biochemistry, Medicine School, UNAM, Mexico City, Mexico; fedebioquimica@bq.unam.mx.
³ Both authors are first authors as they contributed equally to the project.

* Correspondence: suribe@ifc.unam.mx; Tel.: 5555625632

Abstract: *Saccharomyces cerevisiae* mitochondrial unspecific pore (sMUC) is an uncoupling unspecific pore that shares some similarities with the mammalian permeability transition pore (mPTP). When open, both channels deplete ion and proton gradients across the inner mitochondrial membrane. However, the role of mPTP is to reversibly open to protect cells against stress. If mPTP remains stuck in the open position the cell dies. In contrast, sMUC is probably dedicated to deplete oxygen from the medium in order to kill competing organisms. Such O₂ depletion would be better achieved if oxidative phosphorylation is at least mildly uncoupled. Still, when oxidative phosphorylation is needed sMUC should be able to close. To test this, the reversible opening and closing of sMUC in the presence of different effectors was tested in isolated mitochondria from *S. cerevisiae*. Evaluations were conducted at different incubation times, monitoring the rate of O₂ consumption, mitochondrial swelling and the transmembrane potential. It was observed that sMUC did remain reversibly open for minutes. A low energy charge (ATP/ADP) closed the channel. In addition, high Ca²⁺ promoted closing and it was a highly powerful effector.

Keywords: Physiological uncoupling, Yeast mitochondria, Mitochondrial Permeability transition, sMUC, Reversibility.

1. Introduction

Mitochondrial oxidative phosphorylation (OxPhos) involves two coupled processes. First, the exergonic transit of electrons through the redox complexes in the respiratory chain (RC) drives an active transport of protons across the inner mitochondrial membrane to generate a ΔpH [1]. Then the proton gradient is dissipated by the F₁F₀-ATP synthase to produce ATP [1,2]. In the absence of ATP synthesis, ΔpH remains high, decreasing the rate of electron flow [3]. Stalled electrons form free radicals in the different redox centers located in RC complexes that may react spontaneously with O₂ to produce highly motile Reactive Oxygen Species (ROS) [4]. While a small amount of ROS is always produced, an excess of ROS may damage proteins and lipids, leading to aging and even cell death [5,6]

Increasing the rate of electron flow through the respiratory chain decreases ROS production [7]. In the absence of ATP synthesis, the rate of electron flow may be increased by physiological uncoupling [8]. Physiological uncoupling mechanisms include proton sinks, which may be either non-specific or specific for protons [9] and branched respiratory chains, which express alternative redox enzyme that are not sensitive to the
Uncoupling must be tightly controlled through differential expression and/or activity regulation in order to avoid depleting cellular ATP [11].

Saccharomyces cerevisiae is a unicellular eukaryote that displays two strategies to manipulate its environment, which are meant to eliminate other organisms that compete for nutrients: A P-type H⁺-ATPase located in the plasma membrane lowers the external pH to values around 3.0 [12] and in addition its mitochondrial respiratory chain (RC) depletes O₂ from the medium[13]. In these conditions, S. cerevisiae cells survives using an anaerobic fermentative metabolism. Thus, mitochondria from S. cerevisiae must consume O₂ and oxidize NADH + H⁺ at high rates, independently of whether they phosphorylate ATP or not [14,15]. To do this, these mitochondria produce less ATP/O₂ and in addition, a physiological oxidative phosphorylation (OxPhos) uncoupling system is used. Low ATP/O₂ results from the fact that S. cerevisiae mitochondria do not have Complex I, instead they have three type two NADH dehydrogenases, which are not proton pumps [16]. This already leads to a high rate of O₂ consumption. In addition, the S. cerevisiae Mitochondrial Unspecific Channel (sMUC) may open to uncouple OxPhos. This channel is a proton sink, as it allows the passage of molecules up to 1.1 kDa and depletes all ion gradients, ΔpH included [17–19].

The mammalian permeability transition pore (mPTP) is an uncoupling system similar to sMUC[20]. Both sMUC and mPTP are highly effective to equilibrate proton and ion concentrations between the mitochondrial matrix and the cytoplasm [21]. An open sMUC allows the passage of molecules up to 1.1 kDa, while mPTP has a cutoff limit of 1.5 kDa [17,22]. In spite of their functional similarities, the physiological role of each of these channels seems to be different as mPTP opens to protect the cell against ischemia [23], while sMUC is probably designed to deplete O₂ [24]. Also, these channels express different sensitivity to some effectors [25]. Notably, Ca²⁺ closes sMUC while it opens mPTP. In addition, high Pi closes sMUC but opens mPTP [18,26]. Also, ADP closes both channels, but ATP opens sMUC while it closes mPTP [27–30].

An important feature of physiological uncoupling systems is that they have to be tightly controlled to avoid depleting cellular ATP [31]. Unicellular organisms and some tissues such as cardiac muscle, where mitochondria are often exposed to large variations in metabolic demand or O₂ and solute concentrations, seem to undergo frequent reversible opening and closing of PTP. However, after long incubation times, the pore may be stuck in the open position, leading to cell death [32–34]. Thus, it was decided to evaluate for how long sMUC remains reversible and to determine which important effectors, such as adenine nucleotides of Ca²⁺ continue to control opening and closure of this channel after long incubation times.

2. Results
2.1 Effects of ADP/ATP on sMUC.

Both sMUC and mPTP are considered equivalent, except for some differences in effectors, e.g., in contrast to PTP, both Ca²⁺ and Pi close sMUC. Adenine nucleotides are among known effectors of both mPTP and sMUC: in mPTP Pi opens the channel while ATP and ADP close it. In yeast ATP opens sMUC, while ADP and Pi close them[34–37]. Thus, to test sMUC reversibility we alternatively added ATP and ADP in the presence of different Pi concentrations and tested the ability of these molecules to counteract the effects of each other (Fig. 1). In agreement with data in the literature, a slow rate of oxygen consumption was observed at 2.0 mM Pi (Fig. 1-A, trace a), suggesting that sMUC was closed[18,19]. Under these conditions, the addition of different concentrations of ATP increased the rate of oxygen consumption (Fig. 1-A, traces b to e). In contrast, at 0.1 mM Pi the rate of oxygen consumption was high (Fig. 1-B trace a), suggesting that sMUC was open[19,38]. Here, addition of increasing concentrations of ADP inhibited the rate of
Oxygen consumption, suggesting that ADP closed sMUC, i.e., reverted the open state promoted by low Pi (Fig. 1B traces b to e).

Figure 1. Rates of O2 consumption of isolated yeast mitochondria.

![Figure 1. O2 consumption rates of isolated yeast mitochondria. Effects of phosphate (Pi), ATP and ADP. A: Reaction mixture: 0.6 M mannitol, 5 mM MES, pH 6.8, 0.1 mM KCl, 0.5 mM MgCl2, 2 μL/mL ethanol, 0.1 mM phosphate. ADP additions were: a) 2 mM; b) 1.5 mM; c) 1 mM; d) 0.5 mM and e) none ADP. B: Reaction mixture: as in A, except 2 mM phosphate. ATP additions were: a) none ATP; b) 0.5 mM; c) 1 mM; d) 1.5 mM and e) 2 mM.](image)

Oxygen consumption results confirmed that sMUC was regulated by Pi, ADP and ATP. To further explore this phenomenon, it was decided to determine whether changes on ΔΨ were consistent with alternating PT states in response to sequential additions of ADP and ATP (Fig. 2). At low Pi, sMUC was open (Fig. 2A trace a) and ΔΨ was low; here, addition of 2 mM ADP closed sMUC increasing ΔΨ (Fig. 2A trace b). Then the ADP-mediated increase in ΔΨ was reversed by different [ATP], leading to a second depletion of ΔΨ (Fig. 2A, traces c,d,e). The opposite experiment was also performed: when in the presence of high Pi, sMUC was closed as evidenced by a high ΔΨ (Fig. 2B). Here ATP addition led to opening of sMUC decreasing ΔΨ; (Fig. 2B, trace b). Then the addition of different [ADP] promoted a new rise in ΔΨ, suggesting sMUC closed again (Fig. 2B traces c,d,e). Thus, the alternative addition of ADP and ATP resulted in a reversible sequence of opening/closing of sMUC (Fig. 2).

Figure 2. Effect on the mitochondrial transmembrane potential.
Figure 2. Effect of the sequential addition of ATP and ADP on the mitochondrial transmembrane potential ($\Delta \Psi$). Reaction mixture as in Fig. 1, except 15 $\mu$M safranine-O and Pi. Mit: 0.5mg/mL mitochondria; 0.1 mM Pi (a-e) and 1 mM ADP (b-e). ATP additions were: a) 0 mM; b) 0.5 mM; c) 1 mM d) 1.5 mM; e) 2mM. B 2 mM Pi (a-e) and 1 mM ATP (b-e). ADP additions were: a) 0 mM; b) 0.5 mM; c) 1 mM d) 1.5 mM; e) 2mM. Measurements were conducted in a Ollis/Aminco spectrophotometer in Dual mode at 511-533nm.

Another parameter commonly used to follow PT is mitochondrial swelling. At high Pi or high ADP, sMUC was closed and the rate of swelling was slow (Fig. 3, traces a). At low Pi or high ATP, sMUC was open and added K$^+$ ion entered the mitochondrial matrix causing a rapid rate of swelling (Fig. 3, traces b) [39]. Alternation between opening and closing was followed. In the presence of 2.0 mM Pi and 1 mM ADP, swelling was slow (Fig. 3A, trace a) and then, addition of different [ATP] caused increasing rates of swelling, suggesting opening of sMUC (Fig. 3A traces c, d and e). In the opposite sense, at 0.1 mM Pi plus 1 mM ATP a rapid rate of swelling was observed (Fig. 3B, trace b), then, addition of different [ADP] inhibited the rate of swelling (Fig. 3-B traces c, d, and e) suggesting that ADP closed sMUC. Therefore, the effect of Pi, ADP or ATP was known in isolation, with these results we observed that the phenomenon of these three effectors; in the same experiment, can open and close making the sMUC dynamic.

Figure 3. Effect on mitochondrial swelling.
To test the reversibility of ScMUC after long incubation times, yeast mitochondria were incubated for several minutes under conditions where the pore was open and then ADP was added. Both the $\Delta \Psi$ and mitochondrial swelling were monitored. When ADP was added after different incubation times, it was observed that the $\Delta \Psi$ recovered and swelling was reverted suggesting that the ScMUC closed, and thus its reversibility was quite robust. (FIG 4A&B)

**Figure 3.** Effect of the sequential addition of ATP and ADP on mitochondrial swelling. A: Reaction mixture, as in Fig. 1, except 0.3 M mannitol. The arrow indicates the addition of 0.1 M KCl. Swelling was measured in an Ollis/Aminco spectrophotometer in Split mode at 560 nm. 0.1 mM Pi (a-e); 1 mM ADP(b-e). ATP: a) 0 mM; b) 0.5 mM; c) 1 mM ; d) 1.5 mM; e) 2mM B: as in Fig. 2 except 2 mM Pi. Additions were (a-e); 1 mM ATP (b-e). ADP: a) 0 mM; b) 0.5 mM; c) 1 mM ; d) 1.5 mM; e) 2mM

The above results indicate that the opening response of ScMUC to the sequential additions of ATP and ADP is reversible for several minutes of incubation. The next question addressed here is whether ScMUC reversibility is observed in the presence of other effectors, so we decided to test the effect of Ca$^{2+}$ and EGTA at different incubation times.

2.2 Effects of Ca$^{2+}$/EGTA additions on ScMUC.

Ca$^{2+}$ withdrawal from the medium opens ScMUC, while addition of Ca$^{2+}$ closes it. This is an efficient signaling system that controls ATP production during different events such as the cell cycle or mating. At short incubation times, the effects of Ca$^{2+}$ and EGTA are fully reversible. To test the reversibility after several minutes when using Ca$^{2+}$ as an effector, mitochondria were incubated in the presence of EGTA for increasing incubation times and then Ca$^{2+}$ was added to promote closing. Measurements of $\Delta \Psi$ and mitochondrial swelling were conducted to evaluate PT reversibility. Yeast mitochondria were incubated in the presence of EGTA for 15, 30 seconds and 1, 2 and 4 min. Then Ca$^{2+}$ was added and the effects were evaluated. At low Pi and in the presence of EGTA, the addition of K$^+$ led to a decrease of $\Delta \Psi$. Then, the addition of Ca$^{2+}$ at the indicated times pro-
moted a recovery of the $\Delta \Psi$. This was observed at all the incubation times tested (FIG 5A&B). When swelling of mitochondria was tested at low Pi and in the presence of EGTA, $K^+$ promoted swelling, which was reverted by Ca$^{2+}$ addition at all the incubation times tested. Thus, addition of Ca$^{2+}$ led to full recovery of $\Delta \Psi$ (FIG 5A) and to reversal of swelling (FIG 5B) indicating that sMUC closed after long incubation times in the open state. Reversal was even more pronounced than the one obtained using ATP/ADP additions.

**Figure 5.** Effect of the addition of Ca$^{2+}$ on the mitochondrial transmembrane potential and mitochondrial swelling

![Figure 5](image)

**Figure 5.** Effect of the addition of Ca$^{2+}$ on the mitochondrial transmembrane potential and mitochondrial swelling. A: Reaction mixture as in Fig. 2. Ca$^{2+}$ 600 $\mu$M additions (upward arrows) were at different times: 30 sec, 1, 2 and 4 min. B: Reaction mixture as in Fig. 3. Where 0.1 M KCl. Additions of 600 $\mu$M Ca$^{2+}$ were at: 30 sec, 1, 2 and 4 min (upward arrows).

### 3. Discussion

*S. cerevisiae* exhibits a high rate of electron flow through the RC that is further accelerated in the presence of glucose [40]. This is reminiscent of the Crabtree effect originally reported by Otto Warburg in cancer cells [40]. Both in cancer cells and in yeast, energy for growth is provided by anaerobic glycolysis [41]. It has been proposed that in tumor cells, the uncoupled, fast rate of $O_2$ consumption is needed to inhibit production of ROS, helping the cells to survive [42,43]. In contrast, in *S. cerevisiae* this does not seem to be the case as these cells deplete $O_2$ thriving in anoxic environments.

The high rate of $O_2$ consumption by *S. cerevisiae* is slightly uncoupled due to different factors. First, yeast mitochondria do not have a proton-pumping respiratory Complex I, as it is substituted by three alternative NADH dehydrogenases (ND2) [15]. ND2s catalyze a much faster electron transfer than Complex I, which has a slow, complicated catalytic cycle. ND2s do not contribute to the protonmotive force so the ADP/O is lower than in other (mammalian) mitochondria. In addition, they express sMUC, a
A system to deplete O$_2$ would confer yeast with an advantage over other organisms as these cells thrive in hypoxic/anoxic conditions. The reversibility of the permeability transition by the alternate addition and withdrawal of calcium has been documented [18]. Mitochondria from yeast do not have a calcium transporter and the slow uptake of this ion may have been a factor in our results [44]. In contrast mammalian cells move Ca$^{2+}$ through a mitochondrial uniporter (MCU) controlling respiration, mitophagy/autophagy, and mitochondrial apoptosis [45].

Unlike mammals, in *S. cerevisiae* cytoplasmic Ca$^{2+}$ transients may last up to 60 minutes, signaling for processes such as mating or during the cell cycle. Perhaps these are the times when the highest mitochondrial ATP production is needed. In addition, external events such as, alteration in osmolarity or the recovery response from starvation once a substrate becomes available [46]. When a haploid type a cell detects alpha-pheromone, it needs to form a large projection designed to reach for an alpha cell nearby. Then, both cells mate and become a diploid, a process that probably requires a large amount on energy. In these circumstances no apoptotic mechanisms are triggered [47]. Instead, Ca$^{2+}$ probably enhances OxPhos. Indeed, Ca$^{2+}$ efficiently closed ScMUC increasing $\Delta\Psi$ and reversing mitochondrial swelling. Thus, in *S. cerevisiae*, PT was alternatively evoked by ATP and reversed by ADP plus Pi. i.e. a decrease in energy charge optimizes OxPhos. In each case, OxPhos is stimulated by either low energy charge (a decrease in ATP/ADP) or an increase in Ca$^{2+}$, which indicates that a large quantity of ATP will be needed.

In mammals, mPTP is related to a stress response. It is interesting to note that training confers heart mPTPs with both, the ability to open and thus avoid overproducing ROS and to close after an ischemic event, re-coupling OxPhos and helping the cell to survive an ischemic event [24,48]. In this regard, it has been observed that during cardiac stress situations, intermittent episodes of reperfusion allow the cell to recover ATP pools and avoid ROS overproduction, greatly improving survival probabilities. This procedure is termed conditioning [49,50].

In *S. cerevisiae*, the opening of the ScMUC probably does not trigger apoptosis [10] but instead seems to help deplete O$_2$. Most likely, the widely different roles that mPTP and ScMUC have is a classical example of exaptation, where two similar structures with similar mechanisms of action serve different functions in their respective organisms.[51–54] This is further complicated by the fact that the molecules constituting each channel are still undefined.

4. Materials and Methods

**Reagents:** All chemicals were analytic grade. Mannitol, MES hydrate, D-Galactose, TEA (triethanolamine), ADP, ATP, safranine-O, oligomycin were from Sigma Chem Co. Bovine serum albumin from Probulmin TM. Bacto-peptone and yeast extract from MCD LAB. H$_3$PO$_4$, KCl and MgCl$_2$ were from J.T. Baker.

**Yeast strain.** Experiments were conducted using either a commercial strain of baker’s yeast *Saccharomyces cerevisiae* (“La Azteca, S. A.” Mexico City) was used [26] or a laboratory strain W303 (MATa; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100). Results were similar for each strain. Each strain was maintained in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% bacto-agar) plate cultures. Cells were grown as follows: pre-cultures were prepared immersing a loophole of yeast into 100 mL YPD and incubated for 24 hours under continuous agitation in an orbital shaker (New Brunswick Scientific) at 250 rpm in a constant-temperature room (30 °C). Then each 100
mL flask was used to inoculate 900 mL YPGal of (1% yeast extract, 2% bacto-peptone and 2% galactose). Incubation was continued for 48 h under the same conditions.

**Isolation of yeast mitochondria.** After incubation, yeast was centrifuged (5000 xg for 5 min, twice) and resuspended in 0.6 M mannitol, 5 mM MES, 0.1% bovine serum albumin, pH 6.8 (TEA). The cell suspension was mixed with 50% (v/v) of 0.5mm diameter glass beads and disrupted in a Bead Beater and mitochondria were isolated from the homogenate by differential centrifugation as previously described [55]. The concentration of mitochondrial protein was determined by Biuret [56]. In all assays 0.5 mg mitochondrial protein/mL. In all experiments the mitochondrial sample was pre-incubated for 5 min with oligomycin (4 μg per mg protein).

**Oxygen consumption.** Experiments were conducted using a Clark electrode (Oximeter model 782, Warner/Strathkelvin Instruments) in a water-jacketed chamber. Temperature was kept at 30°C using a water bath (PolyScience 7 L). Total volume 1.0 mL. The reaction mixture for yeast mitochondria was 0.6 M mannitol, 5 mM MES (TEA), pH 6.8 plus 0.1 M KCl, 0.5 mM MgCl₂ and 2 μL/mL ethanol. Where indicated, oligomycin 4 μg/mg prot. preincubating for 5 min.

**Transmembrane potential.** ΔΨ was determined as described by Akerman and Wikström [57], following the changes in absorbance of safranine-O at 511-533 in a double beam Aminco-Ollis spectrophotometer in dual mode. The concentrations of ATP, ADP, Ca²⁺ and EGTA used are indicated under each figure. At the end of each trace, the collapse in ΔΨ was induced by addition of 6 μM CCCP.

**Mitochondrial swelling.** Experiments were performed by following the decrease in absorbance of a mitochondrial suspension in a DW2000 Ollis/Aminco spectrophotometer in split mode at a wavelength of 540 nm [37].

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