In *Saccharomyces cerevisiae* Fructose-1,6-bisphosphate contributes to the Crabtree effect through closure of the mitochondrial unspecific channel

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In *Saccharomyces cerevisiae* Fructose-1,6-bisphosphate contributes to the Crabtree effect through closure of the mitochondrial unspecific channel.

In *S. cerevisiae* F1,6BP promotes the Crabtree effect.

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Abstract

In *Saccharomyces cerevisiae* addition of glucose inhibits oxygen consumption, i.e. *S. cerevisiae* is Crabtree-positive. During active glycolysis hexoses-phosphate accumulate, and probably interact with mitochondria. In an effort to understand the mechanism underlying the Crabtree effect, the effect of two glycolysis-derived hexoses-phosphate was tested on the *S. cerevisiae* mitochondrial unspecific channel ($_{Sc}$MUC). Glucose-6-phosphate (G6P) promoted partial opening of $_{Sc}$MUC, which led to proton leakage and uncoupling which in turn resulted in, accelerated oxygen consumption. In contrast, fructose-1,6-bisphosphate (F1,6BP) closed $_{Sc}$MUC and thus inhibited the rate of oxygen consumption. When added together, F1,6BP reverted the mild G6P-induced effects. F1,6BP is proposed to be an important modulator of $_{Sc}$MUC, whose closure contributes to the “Crabtree effect”.

Keywords: Fructose-1,6-bisphosphate, Glucose-6-phosphate, Crabtree effect, Mitochondria, *Saccharomyces cerevisiae*, permeability transition.

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1. Introduction

In “Crabtree positive” yeast, the addition of glucose both increases glycolysis and inhibits the rate of oxygen consumption [1, 2]. It has been proposed that glucose addition induces a rapid metabolic switch from a gluconeogenic /respiratory metabolism to a fermentative mode [3]. The Crabtree effect and the Warburg effect are different in that the Crabtree effect is immediate and reversible, while the Warburg effect is established at longer times, after the expression of different proteins that lead to its irreversibility. Both phenomena have been observed in tumor cells[1]. The Crabtree effect is triggered by different metabolic signals [2, 4, 5]. Among these is the accumulation of the glycolytic intermediaries glucose-6-phosphate (G6P) [6-10 mM], and fructose-1,6-bisphosphate (F1,6BP) [5-10mM] [6-8]. Glycolysis-derived accumulation of hexoses-phosphate complements other known signaling molecules such as fructose-2,6-biphosphate [9].

The Crabtree effect is observed in tumor cells [10, 11], highly proliferating non-tumor cells [11], some yeast species [12] and some bacteria [13]. In regard to the mechanism underlying the Crabtree effect, a competition between glycolysis and oxidative phosphorylation for ADP or Pi has been proposed [14-16]. The mechanism underlying the Crabtree effect is still elusive, although inhibition of complex III and complex IV by F1,6BP has been reported [17].

Most Crabtree positive cells accumulate F1,6P and G6P, which seem to modulate both glycolysis and oxidative phosphorylation [17-19]. Indeed, G6P and

F6P activate the mitochondrial respiratory complex III, while F1,6BP inhibits the activity of both complex III and IV [17].

In the yeast *Saccharomyces cerevisiae,* oxidative phosphorylation is strongly regulated by the mitochondrial unspecific channel ($_{Sc}$MUC) [20, 21]. MUCs have been observed in animals, plants and yeast [22, 23]. MUCs opening, known as the permeability transition (PT), allows the passage of molecules up to 1.5 kDa [23-27], which results in mitochondrial swelling, transmembrane potential depletion and even rupture of the outer membrane [28]. It has been suggested that PT is physiological and reversible and that its main function is to eliminate cations or to partially uncouple the respiratory chain to prevent ROS overproduction [22, 24, 29-31]. ATP, low Pi and the rapid flow of electrons through the respiratory chain promote opening of $_{Sc}$MUC [20, 32, 33], while Pi, Ca^{2+} and Mg^{2+} close it [26].

In order to determine the mechanism by which G6P and F1,6BP control mitochondrial metabolism and whether these molecules contribute to the Crabtree effect, their effects of these hexoses-phosphate on $_{Sc}$MUC were tested. It was observed that G6P opens $_{Sc}$MUC while F1,6BP closes it. When added together, the F1,6P effect dominated. We propose that the closing of the $_{Sc}$MUC by F1,6BP inhibits the rate of oxygen consumption in the resting state through the tight coupling of mitochondria, i.e. F1,6BP is a Crabtree effect promoter.

2. Material and methods.

2.1 Materials

All chemicals were of the highest purity commercially available. Fructose-1,6-bisphosphate, MES, mannitol, triethanolamine, O-safranine, trizma-base, dextrose, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and glucose-6-phosphate, were from Sigma-Aldrich Co. (St. Louis, MO), $(NH_4)_2SO_4$, D-lactic acid and ethanol were from J.T. Baker S.A. de C.V. (Xalostoc, México), yeast extract and gelatin peptone were from Bioxon Dickinson, S.A. de C.V. (Cuautitlán Izcalli, México), KH_2PO_4, KCl, and phosphoric acid were from Química Suastes S.A. de C.V. (Tlahuac, México), BSA type V was from Research Organics (Cleveland, OH).

2.2 Growth conditions

An industrial strain of baker's yeast "yeast foam" (YF) and the *Kluyveromyces lactis* strain 12/8 were used [34]. A 75 mL preculture in YPD (1% yeast extract, 2% gelatin peptone, 2% dextrose) was maintained for 8 h at 30°C under agitation at 250 rpm. Subsequently, the pre-culture was added to 1L of YPLac (1% yeast extract, 1% gelatin peptone, 0.12% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 and 2% lactic acid, pH= 5.5) and incubated overnight. The cells were washed twice with distilled water by centrifugation in a F14 6x250y Sorvall rotor at 3800 xg for 5 min.

2.3. Isolation of mitochondria

Mitochondria were obtained by homogenization and differential centrifugation [35]. Briefly, cells were suspended 50% w/v in mitochondrial buffer (0.6 M mannitol, 5 mM MES pH 6.8 TEA) plus 0.1% BSA and were homogenized in a Bead Beater

using 0.5 mm glass beads [36]. The homogenate was centrifuged in a F21-8x50y Sorvall rotor at 1 017 xg for 5 min. Then the supernatant was recovered and centrifuged at 10 700 xg for 10 min. The pellet was suspended in mitochondrial buffer containing BSA, and centrifuged at 3 600 xg for 5 min. The supernatant was recovered and centrifuged at 17 000 xg for 10 min. The resulting pellet was suspended in a small volume of mitochondrial buffer (without BSA) and protein was determined by biuret [37].

2.4 Oxygen uptake

The rate of oxygen consumption was measured in resting state (State IV), in phosphorylating conditions (state III) and in the presence of the uncoupler CCCP (State U). We used a Strathkelvin Oxymeter model 782 (Warner / Strathkelvin Instruments) with a Clark type electrode immersed in a 1 ml chamber with a water bath (PolyScience model 9000, USA) at 30°C. The reaction mixture was 0.6 M mannitol, 5 mM MES, pH 6.8 (TEA), 10 mM KCl and 2 µl/ml ethanol. Pi concentrations used are indicated in the legends of the figures and tables. Mitochondrial protein concentration was 0.25 mg/ml.

2.5 Mitochondrial swelling.

The K^+-mediated mitochondrial swelling was determined at room temperature. The reaction mixture was 0.3 M mannitol, 5 mM MES, pH 6.8 (TEA) and 2 µl/ml, ethanol. Swelling was started with 20 mM KCl as indicated. The absorbance

changes were measured at 540 nm in a DW 2000 Aminco spectrophotometer in split mode equipped with a magnetic stirrer [38]

2.6 Transmembrane potential

The ΔΨ was determined spectrophotometrically using a DW2000 Aminco spectrophotometer in dual mode. The reaction mixture was 0.6 M mannitol, 5 mM MES pH 6.8, 10 mM KCl, 2 µl/ml ethanol and 15 µM O-safranine. Absorbance changes were followed at 511–533 nm [39].

3. Results and Discussion.

G6P increases, while F1,6BP inhibits the rate of oxygen consumption through direct inhibition of the cytochrome complexes III and IV [17]. However, a possible additional effect on $_{Sc}$MUC has not been explored. The opening of $_{Sc}$MUC accelerates oxygen consumption through uncoupling of oxidative phosphorylation [20, 24, 25, 40]. By contrast, when $_{Sc}$MUC is closed, oxygen consumption decreases [41]. Therefore, it was decided to explore in isolated yeast mitochondria the effect of G6P and F1,6BP on the rate of oxygen consumption (Table 1).

G6P was tested at concentrations of 2 to 20 mM in mitochondria where the $_{Sc}$MUC was fully open (0.1 mM Pi), partially open (1 mM Pi) or fully closed (4 mM Pi). When $_{Sc}$MUC was fully open, G6P had no effects (Results not shown). In mitochondria with partially closed $_{Sc}$MUC, G6P increased the rate of oxygen consumption in the resting state IV while the uncoupled state was mildly

accelerated only at the highest concentrations tested. These effects led to a mild decrease in the U/IV quotient (Table 1). In the conditions where $_{Sc}$MUC was fully closed, only the highest concentrations of G6P resulted in a small increase in state IV respiration, while the uncoupled rate did not change significantly. Thus, a small decrease in the U/IV quotient was observed at the highest G6P concentrations tested (Table 1). In all cases, the effects of G6P on the phosphorylating state III were similar to those observed in the uncoupled state (Result not shown). The results suggest that at the tested concentrations, G6P has a mild uncoupling effect.

F1,6BP was tested at concentrations of 2 to 20 mM under conditions where $_{Sc}$MUC was fully open (0.1 mM Pi) or fully closed (4 mM Pi) (Table 2). In mitochondria with an open $_{Sc}$MUC, F1,6BP inhibited both state IV and the uncoupled state, increasing the U/4 from 1.0 in the totally uncoupled control to 2.0 at 6 mM. In mitochondria with a closed $_{Sc}$MUC, F1,6BP also decreased the rates of respiration both in state IV and in the uncoupled state. The U/IV remained constant up to 6 mM F1,6BP, and it decreased at 10 and 20 mM F1,6BP (Table 2). The decrease in the rate of oxygen consumption in state IV respiration and the increase in the U/4 quotient indicated that F1,6BP is a coupling agent. Thus, the oxygen consumption results (Tables 1 and 2) indicate that G6P and F1,6BP are $_{Sc}$MUC effectors, and that while the former is a mild uncoupler, the latter is an efficient coupling agent even at low concentrations.

When $_{Sc}$MUC is open, mitochondria swell upon K^+ addition. Thus, to further investigate whether G6P and F1,6BP modulate $_{Sc}$MUC, swelling was measured. As expected from the oxygen consumption results, G6P had no effect on the opening

of $_{Sc}$MUC (Result not shown). However, in the presence of a partially closed $_{Sc}$MUC (1 mM Pi) a slight rate of swelling was observed which increased mildly at higher G6P concentrations (Fig. 1A). In mitochondria where the $_{Sc}$MUC was closed, K^+-mediated swelling was not observed and G6P did not have any effects (Fig. 1B).

The data in Table 2 suggest that F1,6BP is a coupling agent. To determine whether this effect may be related to $_{Sc}$MUC, we added increasing concentrations of F1,6BP to mitochondria with open $_{Sc}$MUC (0.1 mM Pi). In the absence of F1,6BP, mitochondrial swelling was large and rapid (Fig. 2A). Then, as the F1,6BP concentration increased, swelling decreased, becoming negligible at concentrations of 4 mM and above (Fig. 2A trace f). At 4 mM Pi, where $_{Sc}$MUC was closed, no swelling was detected neither in the control nor at any F1,6BP concentration (Fig 2B). Both oxygen consumption and mitochondrial swelling experiments indicate that F1,6BP promotes closure of $_{Sc}$MUC, leading to coupling of oxidative phosphorylation.

The transmembrane potential decreases upon opening of $_{Sc}$MUC [25], and thus the effect of G6P and F1,6BP on this parameter was also tested. In mitochondria with partially closed $_{Sc}$MUC, G6P depleted the already-low transmembrane potential (Fig. 3A). Under conditions where $_{Sc}$MUC was closed, different concentrations of G6P decreased the transmembrane potential (Fig. 3B). When assaying F1,6BP effects on mitochondria with fully open $_{Sc}$MUC (Fig. 4A), the transmembrane potential increased with increasing F1,6BP concentration, reaching the highest value at 10 mM (Fig. 4A, trace e) and 20 mM (Fig. 4A, trace

f). Under closed $_{Sc}$MUC conditions, a biphasic effect was observed, where a slight hyperpolarization was observed at 6 (Fig. 4B trace d) and 10 mM (Fig. 4B trace e) whereas a modest decrease in the transmembrane potential was observed at 20 mM F1,6BP (Fig. 4B trace f).

Both phosphate hexoses tested here seem to induce opposite effects. G6P is a mild uncoupler, probably opening $_{Sc}$MUC, while F1,6BP has a strong coupling effect that probably results from closing $_{Sc}$MUC. As both molecules are accumulated simultaneously during active glycolysis, it was decided to test which of their effects predominates when both are present.

To close $_{Sc}$MUC even at low Pi (0.1 mM), 2 mM F1,6BP was added. Under these conditions K^+ addition did not induce swelling (Fig 5A, trace a). Then, increasing G6P concentrations promoted swelling, reaching a maximum at 20 mM G6P. The opposite experiment was performed under partially closed $_{Sc}$MUC conditions (1 mM Pi) and in the presence of 10 mM G6P, where a slight swelling was promoted by K^+ addition. Then, at increasing F1,6BP concentrations inhibition of swelling was observed, suggesting that the $_{Sc}$MUC-closing effect of F1,6BP was much stronger than the $_{Sc}$MUC-opening effect of G6P.

Thus, we propose that the two hexose-phosphates known to accumulate during active glycolysis are effectors of the $_{Sc}$MUC. G6P is a mild uncoupler working through the opening $_{Sc}$MUC while F1,6BP has stronger coupling properties that probably result in $_{Sc}$MUC closure. The effect of the hexose-phosphate

derivatives tested here was observed at lower concentrations than those needed to inhibit respiration.

In mammalians, the mitochondrial permeability transition triggers cell death programs while in turn, a persistently closed MUC results in resistance to apoptosis. Indeed, in rat liver, overexpressed hexokinase seems to interact directly with mitochondria, maintaining MUC in a closed state and thus inhibiting cell death [40-42]. F1,6BP promoted $_{Sc}$MUC closure, therefore inhibiting the permeability transition. In addition, a closed $_{Sc}$MUC inhibits apoptosis and promotes unregulated cell growth, indicating that there may be a causal relationship between tumor cell immortalization and F1,6P accumulation.

If the F1,6BP-mediated inhibition of oxygen consumption by *S. cerevisiae* were related to the Crabtree effect, then Crabtree-negative yeasts should not be inhibited. To test this, we compared the effect of 10 mM F1,6BP on mitochondria isolated from either *S. cerevisiae* or from the Crabtree negative yeast *Kluyveromyces lactis* [43]. As expected from the results shown in Table 2, F1,6BP inhibited the rate of oxygen consumption in mitochondria from *S. cerevisiae* at both 0.1 mM (Fig. 6A) and 1.0 mM Pi (Fig. 6B). By contrast, in mitochondria from *K. lactis,* F1,6BP did not have any effects on the rate of oxygen consumption at either phosphate concentration (Fig. 6). These results strongly support the notion that F1,6BP is a key metabolite that signals closure of $_{Sc}$MUC, triggering the Crabtree effect. Indeed, upon glucose addition, F1,6BP rises to 5-10 mM, which is much higher than the concentration of 0.5 mM F1,6BP needed to maintain $_{Sc}$MUC in a closed state [7]. Furthermore, at 5 mM F1,6BP, additional mitochondrial effects are

observed, such as inhibition of complex III and IV activities in the resting state IV. Further studies are needed in order to elucidate the physiological role of maintaining $_{Sc}$MUC closed and dissect its likely relationship with the Crabtree effect.

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Table 1. Effect of G6P on the rate oxygen consumption under conditions where the *Sc*MUC is partially closed (1 mM Pi) or completely closed (Pi 4 mM).

Pi	G6P (mM)	State IV natgO(min*mgprot)$^{-1}$	Uncoupled state natgO(min*mgprot)$^{-1}$	U/IV
	0	132 ± 17.0	210 ± 19.8	1.6
1 mM	2	138 ± 19.8	198 ± 36.8	1.4
	4	150 ± 31.1	212 ± 39.6	1.4
(*Sc*MUC partially closed)	6	160 ± 11.3	214 ± 14.1	1.3
	10	194 ± 19.8	262 ± 14.1	1.3
	20	234 ± 14.1	280 ± 11.3	1.2
	0	162.9 ± 28.8	310.0 ± 45.4	1.9
4 mM	2	170.8 ± 5.25	278.8 ± 32.9	1.6
	4	196.7 ± 23.5	318.2 ± 23.6	1.6
(*Sc*MUC closed)	6	212.0 ± 18.3	333.9 ± 21.6	1.6
	10	208.7 ± 7.8	326.6 ± 11.0	1.6
	20	231.2 ± 16.5	332.8 ± 40.7	1.4

Reaction mixture: 0.6 M Mannitol, 5 mM MES, pH 6.8 (TEA), 2 µl ethanol/mL, 10 mM KCl and Pi as indicated. Glucose-6-phosphate (G6P) as indicated. Mitochondria (250 µg prot./mL). The uncoupled state was generated using CCCP (1.5 µM).

Table 2. Effect of F1,6BP on the rate of O_2 consumption under conditions where the *Sc*MUC is opened (Pi 0.1 mM) or closed (Pi 4 mM).

Pi	F1,6BP (mM)	State IV natgO(min*mg prot)$^{-1}$	Uncoupled state natgO(min*mg prot)$^{-1}$	U/IV
	0	328.4 ± 29.0	339.2 ± 24.0	1.0
	2	167.6 ± 21.3	257.9 ± 46.0	1.5
0.1 mM	4	143.3 ± 27.3	263.2 ± 25.0	1.8
	6	129.4 ± 4.9	254.0 ± 43.0	2.0
(*Sc*MUC open)	10	142.2 ± 12.5	276.7 ± 66.0	1.9
	20	140.6 ± 21.7	250.4 ± 46.0	1.8
	0	240.7 ± 21.1	452.5 ± 40.3	1.9
4 mM	2	214.2 ± 5.1	408.0 ± 48.5	1.9
	4	229.0 ± 19.4	422.2 ± 57.9	1.8
(*Sc*MUC closed)	6	208.3 ± 24.6	385.9 ± 8.8	1.8
	10	194.1 ± 24.4	357.3 ± 56.0	1.8
	20	192.7 ± 27.6	334.7 ± 28.3	1.7

Reaction mixture as in Table 1, except fructose-1,6-bisphosphate (F1,6BP) as indicated.

FIGURE LEGENDS:

Fig. 1. Effects of G6P on mitocondrial swelling. Reaction mixture: 0.3 M mannitol, 5 mM MES, pH 6.8 (TEA), 2 µL ethanol/mL, mitochondria 250 µg/mL, and Pi as indicated. Swelling was measured spectrophotometrically at 540 nm. The arrow indicates the addition of 20 mM KCl. **A**, Pi 1 mM. **B**, Pi 4 mM. G6P (mM) was: a: 0, b, 2, c:,4, d, 6, e, 10, f, 20.

Fig. 2. Effects of F1,6BP on mitocondrial swelling. Reaction mixture as in Fig 1 except **A**, Pi 0.1 mM. **B**, Pi 4 mM. At the arrow 20 mM KCl was added. G6P was: a: 0, b, 0.25, c, 0.5, d,

1, e, 2, f, 4 mM.

Fig. 3. Effects of G6P on the mitochondrial transmembrane potential. Reaction mixture as in Table 1 (except 15µM safranine-O), 10 mM KCl and Pi as indicated. **A**, Pi 1 mM, **B**, Pi 4 mM. G6P (mM) as follows: a, 0 b, 2, c, 4, d, 6, e, 10, f, 20. Where indicated, Mit, mitochondria (250 µg prot./mL) or CCCP (1.5 µM) were added.

Fig.4. Effects of F1,6BP on transmembrane potential. Reaction mixture as in Fig. 3, except **A**, Pi 0.1 mM and **B**, Pi 4 mM. F1,6BP (mM) was: a, 0 b, 2, c, 4, d, 6, e, 10, f, 20. Mit, mitochondria (250 µg/mL), CCCP (1.5 µM).

Fig. 5. Effects of competition between F1,6BP and G6P on mitochondrial swelling. Reaction mixture: as in Fig. 1. Pi as indicated. **A**, Pi 0.1 mM, 2 mM F1,6BP. G6P (mM) was: a, 0, b, 4, c, 10, d, 20. **B**, Pi 1 mM, 10 mM G6P. F1,6BP (mM) was a, 0, b, 0.5, c, 4, d,10.

Fig. 6. Effect of F1,6BP on the rate of oxygen consumption of isolated mitochondria from *S. cerevisiae* or *K. lactis*. Reaction mixture as in Table 2, except **A**, Pi 0.1 mM; **B**, Pi 1 mM F1,6BP was absent (empty bars) or 10 mM (black bars).* Differences are statistically significant $P<0.01$, based on ANOVA tukey's multiple comparison test.

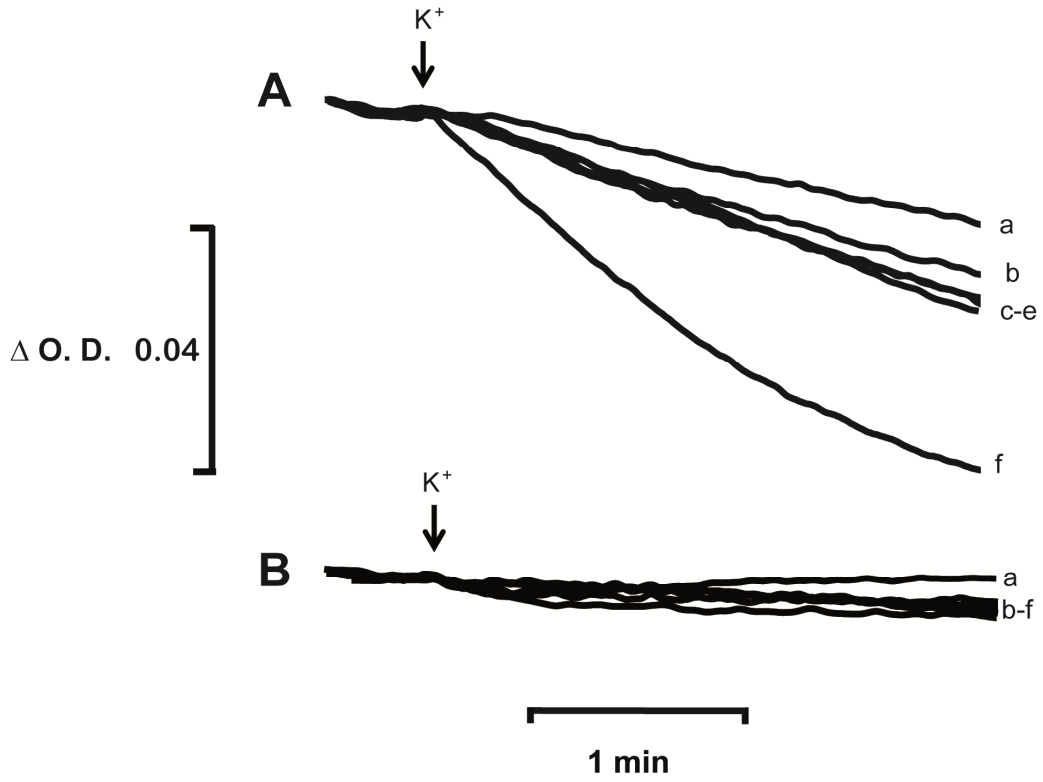


FIG. 1. Rosas-Lemus et al., 2014

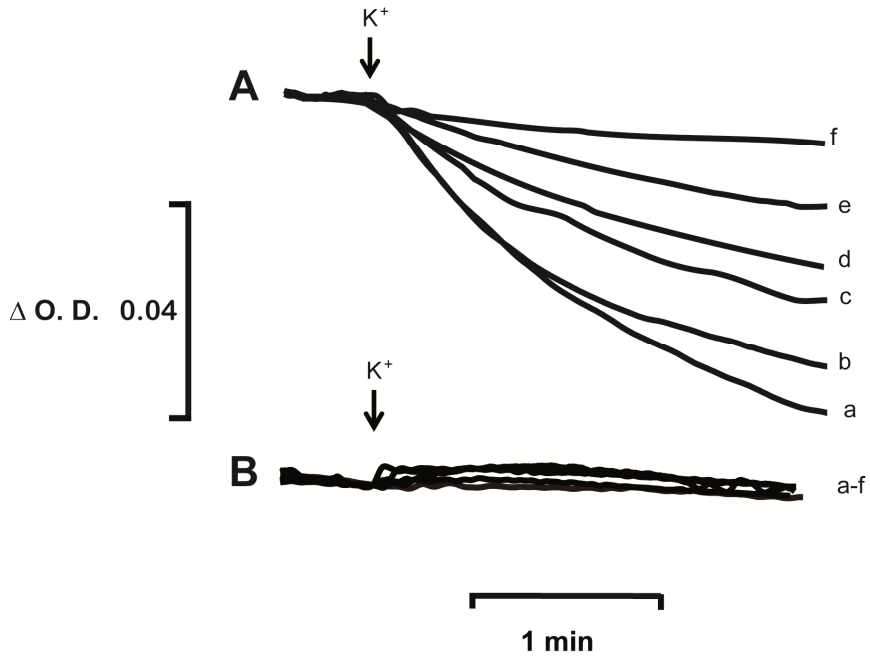


FIG. 2. Rosas-Lemus et al., 2014

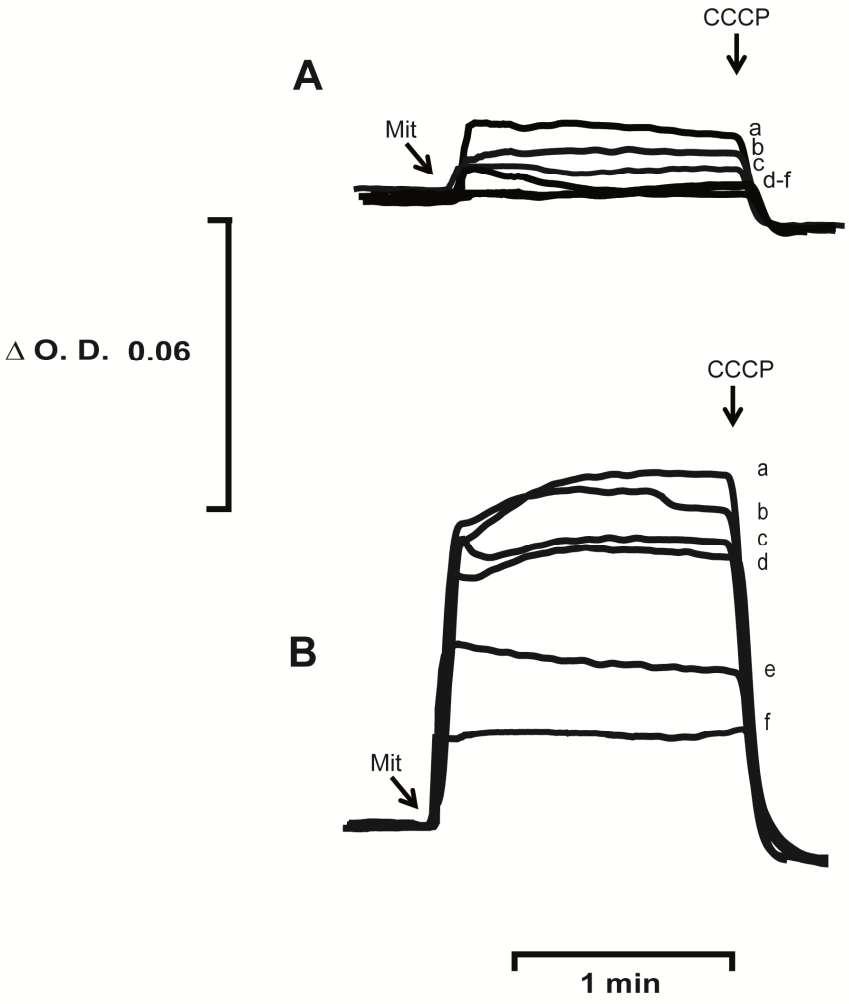


FIG. 3. Rosas-Lemus et al., 2014

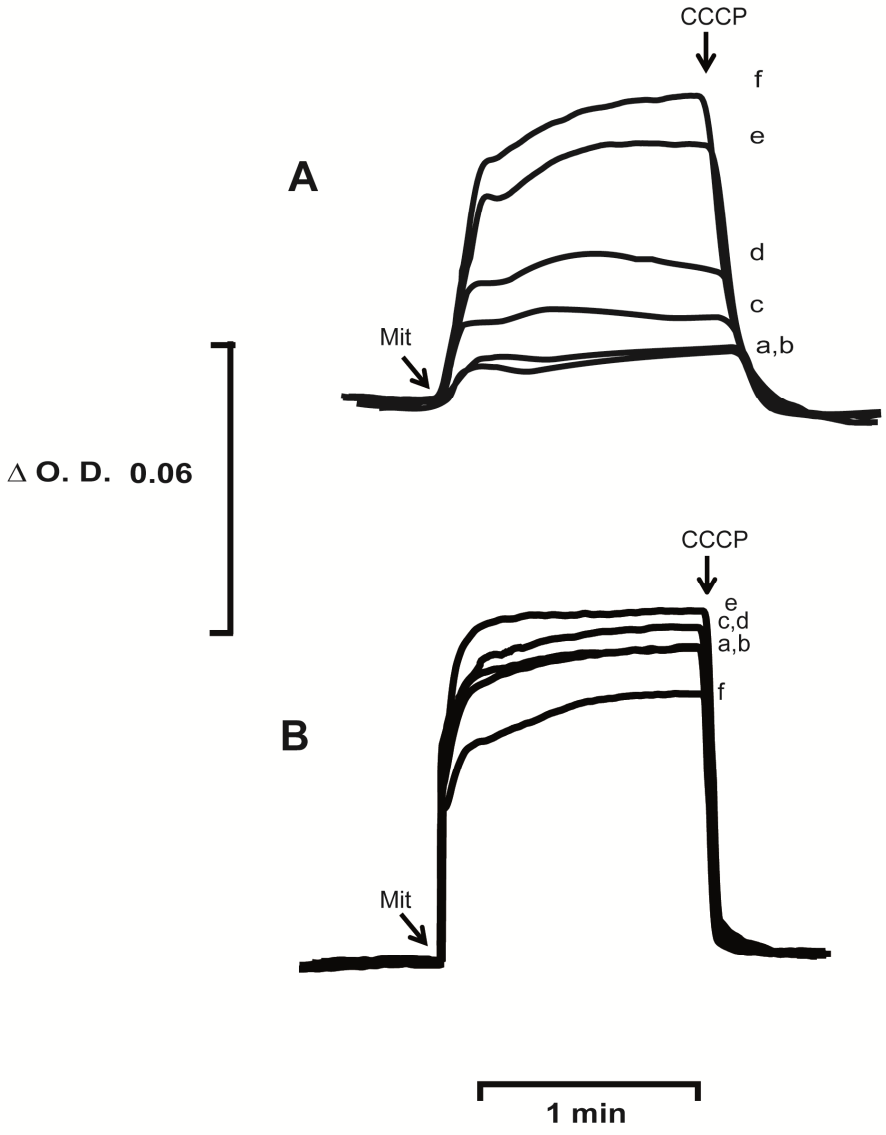


FIG. 4. Rosas-Lemus et al., 2014

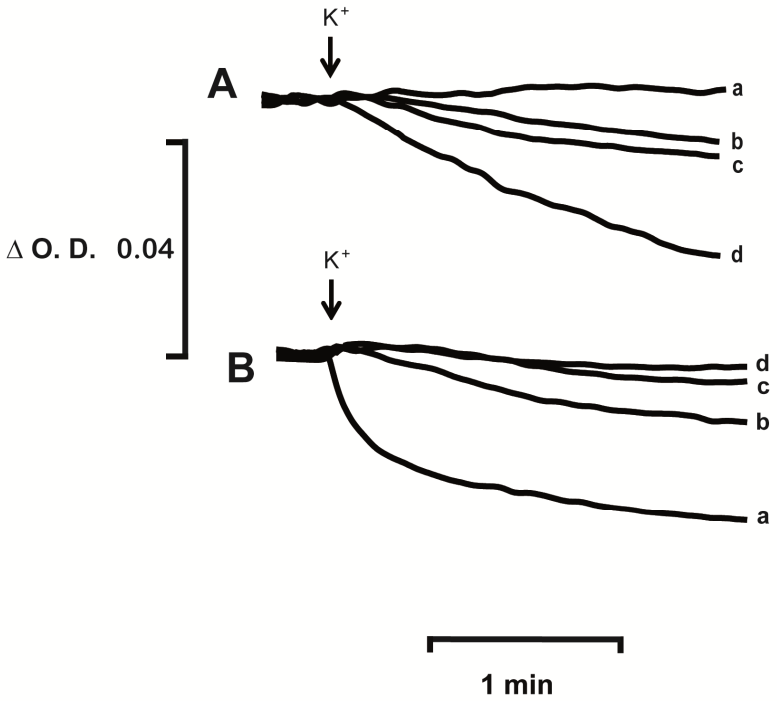


FIG. 5. Rosas-Lemus et al., 2014

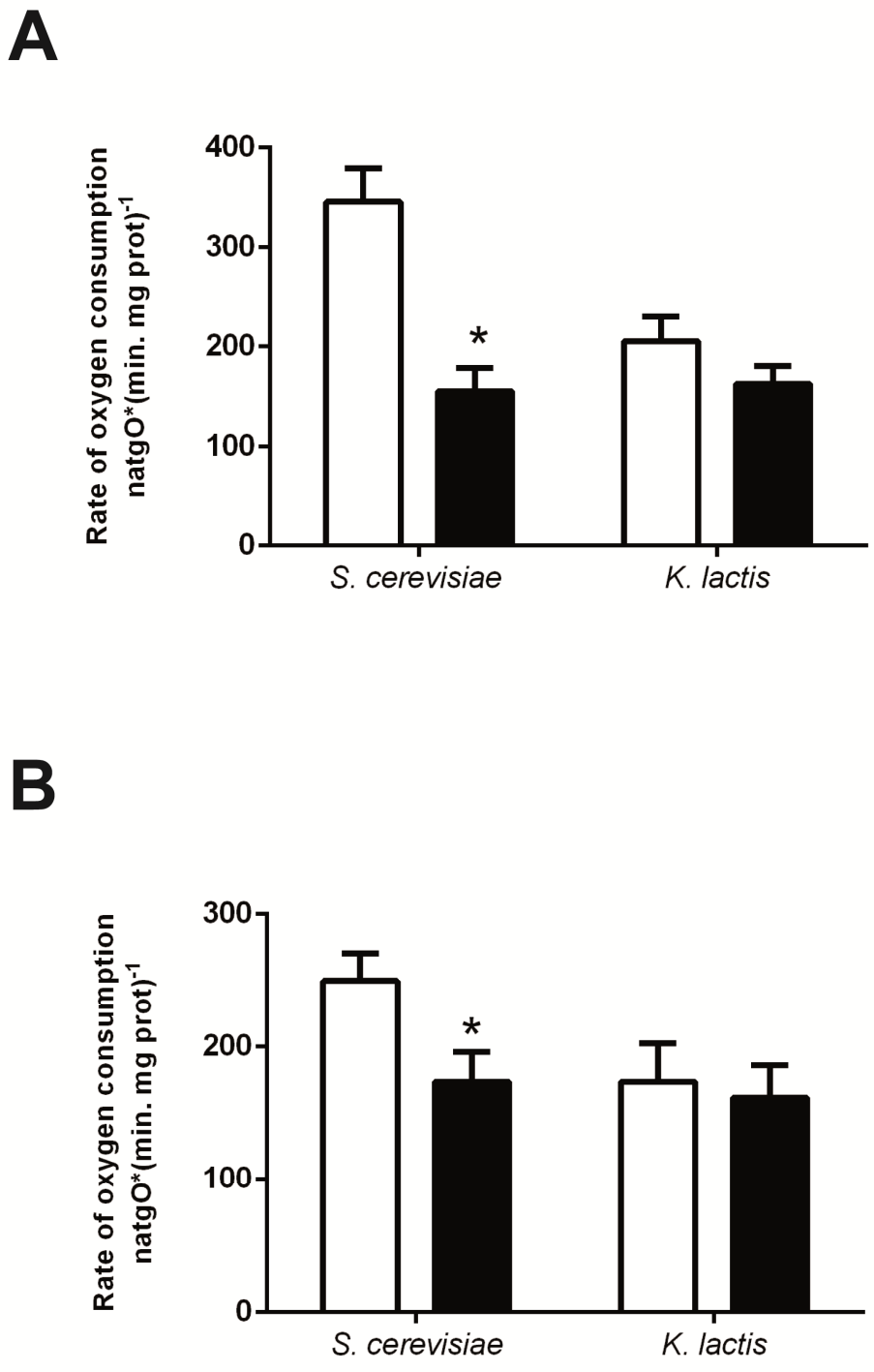
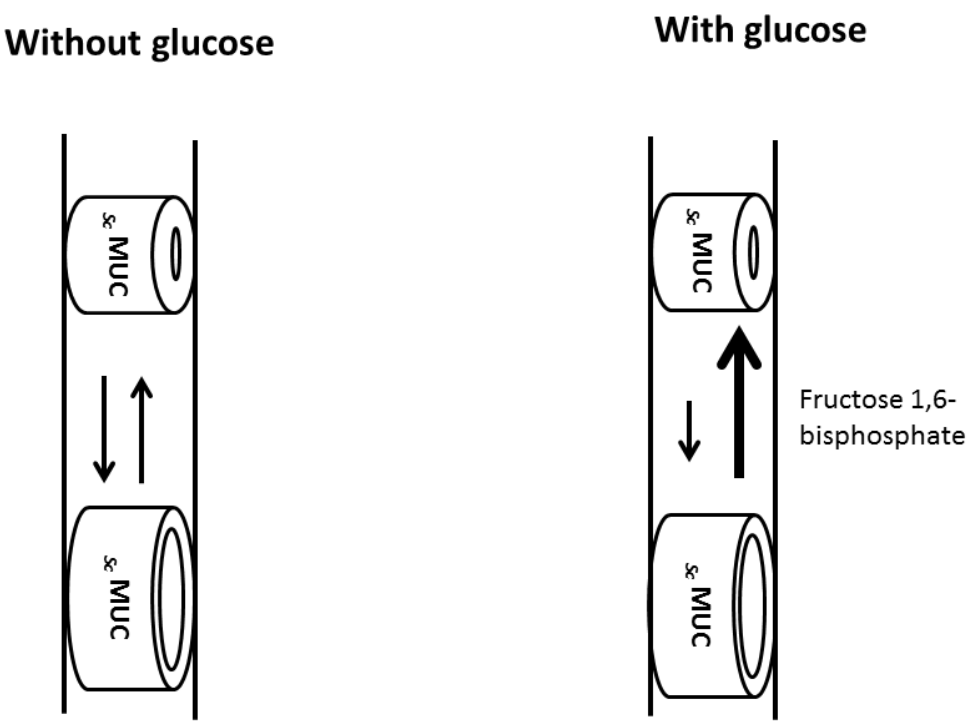


FIG. 6. Rosas-Lemus et al., 2014



Highlights

- In *S. cerevisiae* F1,6 BP promotes closure of $_{sc}$MUC.
- A closed $_{sc}$MUC enhances the mitochondrial membrane impermeability to protons.
- The effect of F1,6BP on $_{sc}$MUC contributes to the Crabtree effect.