J Bioenerg Biomembr (2010) 42:11–19
DOI 10.1007/s10863-009-9264-0

Mitochondria from the salt-tolerant yeast *Debaryomyces hansenii* (halophilic organelles?)

Alfredo Cabrera-Orefice · Sergio Guerrero-Castillo · Luís A. Luévano-Martínez · Antonio Peña · Salvador Uribe-Carvajal

Received: 3 September 2009 / Accepted: 4 December 2009 / Published online: 21 January 2010

Abstract The yeast *Debaryomyces hansenii* is considered a marine organism. Sea water contains 0.6 M Na^+ and 10 mM K^+; these cations permeate into the cytoplasm of *D. hansenii* where proteins and organelles have to adapt to high salt concentrations. The effect of high concentrations of monovalent and divalent cations on isolated mitochondria from *D. hansenii* was explored. As in *S. cerevisiae*, these mitochondria underwent a phosphate-sensitive permeability transition (PT) which was inhibited by Ca^{2+} or Mg^{2+}. However, *D. hansenii* mitochondria require higher phosphate concentrations to inhibit PT. In regard to K^+ and Na^+, and at variance with mitochondria from all other sources known, these monovalent cations promoted closure of the putative mitochondrial unspecific channel. This was evidenced by the K^+/Na^+-promoted increase in: respiratory control, transmembrane potential and synthesis of ATP. PT was equally sensitive to either Na^+ or K^+. In the presence of propyl-gallate PT was still observed while in the presence of cyanide the alternative pathway was not active enough to generate a $\Delta\Psi$ due to a low AOX activity. In *D. hansenii* mitochondria K^+ and Na^+ optimize oxidative phosphorylation, providing an explanation for the higher growth efficiency in saline environments exhibited by this yeast.

Keywords Branched respiratory chain · Divalent cations · Monovalent cations · *Debaryomyces hansenii* · Isolated mitochondria · Permeability transition

A. Cabrera-Orefice · S. Guerrero-Castillo · L. A. Luévano-Martínez · S. Uribe-Carvajal
Department of Biochemistry, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, México

A. Peña
Department of Molecular Genetics, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, México

S. Uribe-Carvajal (✉)
Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apdo. Postal 70-242, Ciudad Universitaria, Coyoacán, Mexico City, Mexico
e-mail: suribe@ifc.unam.mx

Introduction

Debaryomyces hansenii is normally found among the microorganisms populating sea waters and other habitats with low water activity where its halotolerance is an advantage (Norkrans 1966; Norkrans and Kylin 1969). This yeast has found diverse biotechnological applications in recent years, such as production of dairy products and of lytic enzymes of commercial interest (Breuer and Harms 2006). In cheese manufacturing, *D. hansenii* is a choice for starter cultures as it catalyzes proteolysis and lipolysis without fermenting sugars (Fadda et al. 2004). This yeast is also attractive for study due to its ability to grow under extreme conditions such as very low temperatures (Norkrans 1966), widely different pHs (Norkrans 1966; Hobot and Jennings 1981) and high salt concentrations (Norkrans 1966, Norkrans and Kylin 1969; Prista et al. 2005; Ramos 2006). The genome of *D. hansenii* was reported by the génolevure project (Sherman et al. 2004).

At least part of the halotolerance of *D. hansenii* may be due to its potent monovalent cation transporters, present both in the plasma membrane (Hobot and Jennings 1981; Prista et al. 1997; Thomé-Ortiz et al. 1998) and in the vacuole (Montiel and Ramos 2007). However, its proteins and enzymes have to be resistant to salts, because in the

presence of high (0.5–1.0 M) external NaCl the cytoplasmic concentrations of monovalent cations (Na^+ and K^+) reach the hundred mM range (Gónzalez-Hernández et al. 2004). Given the choice, *D. hansenii* accumulates KCl instead of NaCl (Norkrans and Kylin 1969; Thomé-Ortiz et al. 1998) but both cations seem to have the same effects on *D. hansenii*, e.g. the expression of NADP-glutamate dehydrogenase and glutamine synthetase is modified by either Na^+ or K^+; this regulation has not been observed in *Saccharomyces cerevisiae* (Alba-Lois et al. 2004). The Na^+/K^+ effect provides an explanation for the increase in biomass obtained when *D. hansenii* is grown in the presence of high salt concentrations (Prista et al. 1997) and fuels the notion that *D. hansenii* is halophilic and not just osmotolerant (Gónzalez-Hernández et al. 2004).

In the cytoplasm of *D. hansenii,* enzymes are not the only structures exposed to high salts. Organelles also have to deal with concentrations around 0.4 M NaCl/KCl (Neves et al. 1997; González-Hernández et al. 2004). Thus, it would be interesting to analyze the physiology and salt adaptability of each organelle. To do this, we decided to characterize the effect of high salt concentrations on the isolated mitochondria from *D. hansenii*. We theorized that the mitochondrial adaptation to high salt concentrations would have to include strict regulation of cation permeability; otherwise, an electrophoretic cycling of the monovalent cation across the inner mitochondrial membrane would result in the depletion of the transmembrane potential ($\Delta\Psi$) and in uncoupling (Garlid 1988). Indeed, in mitochondria isolated from the yeast *S. cerevisiae* addition of increasing concentrations of K^+ or Na^+ in the presence of low phosphate results in depletion of the $\Delta\Psi$ and a decrease in the synthesis of ATP (Castrejón et al. 1997). By contrast, at high phosphate concentrations monovalent cations do not affect the $\Delta\Psi$, and in these conditions a Mg^{2+}- and quinine-sensitive uptake of $^{86}Rb^+$ is observed (Castrejón et al. 2002).

The permeability transition (PT) occurs when a large mitochondrial unspecific channel (MUC) opens in the inner mitochondrial membrane allowing ions to flow freely into and out from the matrix depleting chemical and electrical gradients (Bernardi et al. 1994; Zoratti and Szabo 1995). The most studied MUCs are those from mammalians and from *S. cerevisiae* (Manon et al. 1998). The mammalian MUC is open by Ca^{2+} in the presence of high phosphate and is closed by ATP and the cyclophylin-D inhibitor cyclosporine-A (Halestrap and Davidson 1990). In *S. cerevisiae* mitochondria, both phosphate (Guérin et al. 1994) and Ca^{2+} (Pérez-Vázquez et al. 2003) prevent the PT. Thus, it was of interest to determine whether Ca^{2+} opens or closes the *D. hansenii* MUC. The structure, properties and regulating factors of the MUCs vary between different species (Pavlovskaya et al. 2007; Brustovetsky et al. 2002; Kusano et al. 2009). However, an organelle normally exposed to high monovalent cation concentrations should be able to block its own conductance to the salts found in high concentrations.

The mitochondria from at least two salinity-adapted yeast species, *Endomyces magnusii* and *Yarrowia lipolytica*, do not seem to undergo PT unless a high concentration of Ca^{2+} plus the Ca^{2+} ionophore ETH129 are added (Deryabina et al. 2004; Kovaleva et al. 2009).

We characterized the PT in isolated mitochondria from *D. hansenii*. We detected a MUC which exhibits a sensitivity to Ca^{2+} and Mg^{2+} which was similar to the *S. cerevisiae* channel. Also, the *D. hansenii* MUC was closed by phosphate, although at higher concentrations than the *S. cerevisiae* channel. In addition, we observed that this channel was different to the MUCs from *S. cerevisiae,* plants or mammalians, in that it was regulated by monovalent cations, i.e. increasing concentrations of Na^+ or K^+ closed the *D. hansenii* mitochondrial channel. It is becoming increasingly evident that the physiological role and the control mechanisms of mitochondrial unspecific channels may be different depending of the species under study.

Materials and methods

Chemicals

All chemicals were reagent grade. Sorbitol, Trizma® base, maleic acid, pyruvic acid, malic acid, NADH, NADP, antifoam emulsion, ADP, safranine-O, hexokinase and bovine serum albumin (BSA) type V were from Sigma Chem Co. (St Louis, MO). Lyophilyzed glucose-6-phosphate dehydrogenase was from Boerhinger-Manheim.

Yeast strains The yeast *Debaryomyces hansenii* strain Y7426 (US Dept. of Agriculture) was used throughout this work. The strain was maintained in Na-YPGal (1% yeast extract, 2% bacto-peptone, 2% galactose, 1 M NaCl and 2% bacto-agar) plate cultures.

Yeast growth media for mitochondrial isolation

Cells were grown as follows: three 100 mL pre-cultures were prepared immersing a loophole of yeast into 100 mL of Na-YPLac (1% yeast extract, 2% bacto-peptone, 2% lactate, pH 5.5 adjusted with NaOH plus NaCl to reach a final 0.6 M Na^+ concentration). Antifoam emulsion 50 µL/L was added to the medium. Flasks were incubated for 36 hours under continuous agitation in an orbital shaker at 250 rpm in a constant-temperature room (30°C). Then,

each 100 mL flask was used to inoculate 750 mL of fresh medium. Incubation was continued for 24 h under the same conditions. At a final optical density of 1.6–2.0, the culture was in the mid to late logarithmic growth phase; the cells were harvested at this stage, before they became resistant to disruption.

Isolation of coupled mitochondria from *Debaryomyces hansenii*

D. hansenii cells were collected and washed by centrifugation and suspended in distilled water. After a second centrifugation, the cells were suspended in ice-cold isolating medium containing 1 M sorbitol, 10 mM maleate, 0.2% bovine serum albumin, pH 6.8 (Tris). The cells were poured into a Bead-Beater 300 mL flask containing 70% v/v 0.5-mm-diameter glass beads. The container was introduced in an ice-jacketed chamber and cells were subjected to four 20 s pulses at 2 min intervals. After homogenization, mitochondria were isolated by differential centrifugation following a protocol described for *S. cerevisiae* mitochondria (Peña et al. 1977). The final mitochondrial pellet was resuspended in 500 μL ice-cold isolation medium.

Some details on the isolation of mitochondria seem pertinent as the procedure is slightly different to the usual procedures (Uribe et al. 1985). Sorbitol was deionized before use (Averet et al. 1998). Osmolarity was 1 OsM; lower osmolarities resulted in uncoupled mitochondria. The same osmolarity was used during all experiments. We did not use zymolyase and/or lyticase to disrupt the membrane due to the rapid inactivation of mitochondria that ensues during the isolation procedure. Instead, we used a Bead-Beater with a large amount (70% v/v) of glass beads. The speed of the Bead-Beater was maintained at half maximum by means of a rheostat, to avoid disruption and uncoupling of mitochondria.

Protein quantification

The concentration of mitochondrial protein was determined by the biuret method (Gornal et al. 1949). Optical absorbance was determined at 540 nm in a Beckman DU-50 spectrophotometer. Protein was determined using BSA as a standard.

Oxygen consumption

The rate of oxygen consumption was measured in the resting state (State IV) and in the phosphorylating state (State III), using an YSI-5300 Oxygraph equipped with a Clark-Type electrode (Yellow Springs Instruments Inc. OH) interfaced to a chart recorder. The reaction vessel was a water-jacketed chamber maintained at 30 °C. Mitochondria, 0.5 mg protein·(mL)$^{-1}$. The reaction mixture was 1 M sorbitol, 10 mM maleate, pH 6.8 (Tris); 10 mM pyruvate-malate was added as a substrate. Final volume was 1.5 mL. The concentrations of phosphate (Pi) and K^+ used are indicated in the legends of each illustration. Stock solutions were 2 M KCl or NaCl and 1 or 0.1 M phosphate buffer pH 6.8 (Tris).

Transmembrane potential ($\Delta\Psi$)

The transmembranal potential was determined using safranine-O, following the absorbance changes at 511–533 nm in a DW2000 Aminco spectrophotometer in dual-wavelength mode (Akerman and Wikström 1976). We used a final concentration of 0.5 mg protein/mL of mitochondria. Yeast mitochondria were assayed in the respiration medium plus 10 μM safranine-O. The concentrations of K^+ and Pi are indicated under each figure. Where indicated, the uncoupler *p*-chloromethoxycarbonylcyanide phenylhydrazone (CCCP) was added to a final concentration of 5 μM.

ATP synthesis

An enzyme–coupled assay system containing 162.5 μg/mL hexokinase, 2 U/mL glucose-6-phosphate dehydrogenase, 20 mM glucose, 1.4 mM $NADP^+$, 200 μM $MgCl_2$ and 10 mM pyruvate-malate, was used to measure the rate of ATP synthesis. The reaction was started by adding 200 μM ADP. The reduction of $NADP^+$ was followed in a DW2C Aminco/Olis spectrophotometer in dual mode at 340–390 nm (Cortés et al. 2000). The NADPH extinction coefficient used was 6.22×10^3 (M·cm)$^{-1}$. The lyophilized enzymes were suspended prior to each experiment as follows: hexokinase was suspended in water to 13 mg/mL and glucose-6-phosphate dehydrogenase was suspended in a 5 mM citrate buffer pH 7 to a final concentration of 200 U/mL. Oligomycin (10 μg/mg prot) was used to determine the basal ATP synthesis; which was subtracted from the experimental data. The concentrations of Pi are indicated in the legend to the figure.

Results and discussion

In *D. hansenii* mitochondria oxygen consumption is coupled by phosphate and by monovalent cations

In all cases, the isolated mitochondria from *D. hansenii* exhibited the same sensitivity to KCl or NaCl. Thus, we are presenting mainly the results obtained with KCl. We chose to show the K^+ effect because in the presence of both cations *D. hansenii* prefers to concentrate K^+; faster and with more affinity than Na^+ (Thomé-Ortiz et al. 1998; González-Hernández et al. 2004). The isolated mitochon-

dria from *D. hansenii* exhibited a slow rate of oxygen consumption both in state IV and in state III, suggesting that the respiratory chain needed the presence of a monovalent cation in order to work at full speed (Table 1). The role of K^+ as an activator of the respiratory chain of mitochondria from yeast (Uribe et al. 1991) and mammalians (Peña et al. 1964; Gómez-Puyou et al. 1969; Gómez-Puyou and Tuena de Gómez Puyou 1977) has been described. The respiratory control (RC) is a measure of the 'integrity' of mitochondria preparation and coupling efficiency between the respiratory chain and the F_1F_0-ATP synthase (Nicholls and Ferguson 2001). In *D. hansenii* it was observed that at 0.4 mM Pi mitochondria were uncoupled as evidenced by a RC of 1.0 (Table 1). Then, addition of different concentrations of K^+ led to higher rates of oxygen consumption and to an increased RC (Table 1) as follows: At 0 to 20 mM K^+, RC was 1.0. However, at 50 mM K^+ RC was 1.33 and increased to 1.73 at the maximum K^+ concentration tested (75 mM) (Table 1). When the phosphate concentration was raised to 4.0 mM, the rates of oxygen consumption were higher (Table 1) and a RC of 1.27 was observed in the absence of K^+ (Table 1). Then, as different K^+ concentrations were added, the RC increased such that at 75 mM K^+, RC=2.29 (Table 1). In the presence of 10 mM Pi, the rates of oxygen consumption were similar to those obtained at 4 mM Pi (Table 1) and the RC was 2.14 in the absence of K^+, and increased only slightly at the K^+ concentrations tested (Table 1), i.e. 10 mM Pi closed the channel without K^+. Even though Pi has the same coupling effect in *D. hansenii* mitochondria as in those from *S. cerevisiae* (Manon and Guérin 1997), the *D. hansenii* organelles need ten times more Pi to close the unspecific channel. In isolated mitochondria from *S. cerevisiae* (Gutiérrez-Aguilar et al. 2010) or mammalians (Leung and Halestrap 2008) the Pi site has been located in the inner membrane and it has been tentatively identified as the phosphate carrier.

In *D. hansenii* mitochondria, opening of a Pi/K^+-sensitive unspecific channel results in depletion of the transmembrane potential ($\Delta\Psi$)

The oxygen consumption data suggested that there is a MUC in *D. hansenii*. Another yeast species, *S. cerevisiae* contains a MUC which is closed by 1–2 mM phosphate (Manon and Guérin 1997). Thus, in order to further explore the sensitivity of the putative MUC from *D. hansenii* mitochondria to Pi, the electric transmembrane potential ($\Delta\Psi$) was measured in the presence of 0.4, 4.0 and 10 mM Pi (Fig. 1). At 0.4 mM Pi, the $\Delta\Psi$ was low and unstable (Fig. 1a, trace a). Then in the presence of 4.0 mM Pi the $\Delta\Psi$ became higher and gained stability (Fig. 1b, trace a) and it reached a high, stable $\Delta\Psi$ at 10 mM Pi (Fig. 1c, trace a) or higher (Result not shown). These results suggested that *D. hansenii* contain a permeability transition pore similar to that observed in *S. cerevisiae* mitochondria, although the organelle from *D. hansenii* requires a higher Pi concentration to close. The oxygen consumption effects reported in Table 1 also suggested that Pi and monovalent cations exhibit synergistic effects. Thus, to further explore the monovalent cation effect, the $\Delta\Psi$ was measured at each of the Pi concentrations tested and in the presence of increasing K^+. At 0.4 mM Pi where the $\Delta\Psi$ was unstable, increasing concentrations of K^+ resulted in higher, more stable $\Delta\Psi$, indicating that at the low Pi concentrations the monovalent cation aided to seal the unspecific channel (Fig. 1a, traces b to e). At 4 mM Pi, the $\Delta\Psi$ was low in the absence of K^+ and increased with K^+ concentrations. This result probably indicates that Pi was able to partially close the channel, although K^+ was still needed to achieve full closure (Fig. 1b). At 10 mM Pi, the highest concentration tested, MUC was completely closed and the monovalent cation did not have further effects (Fig. 1c).

Table 1 Effect of phosphate (Pi) and K^+ on the rate of oxygen consumption and respiratory control of isolated mitochondria from *Debaryomyces hansenii*

	0.4 mM Pi			4.0 mM Pi			10.0 mM Pi		
[K^+] (mM)	IV	III	RC	IV	III	RC	IV	III	RC
0	75±6	75.0±6	**1.0**	130±9	165±8	**1.27**	129±6	277±9	**2.14**
10	79±3	79.0±3	**1.0**	127 ± 10	191 ± 13	**1.51**	127±6	273 ± 14	**2.16**
20	79±3	79.0±3	**1.0**	128±8	220 ± 16	**1.72**	129±6	281 ± 12	**2.18**
50	127±3	168±5	**1.33**	122 ± 6	249±4	**2.05**	124±5	285±3	**2.31**
75	127±3	219 ± 1	**1.73**	114±9	261 ± 9	**2.29**	131 ± 7	293±5	**2.24**

The rates of oxygen consumption in resting state (IV) and phosphorylating state (III) are expressed in natgO_2(min·mg prot)$^{-1}$. The respiratory control (RC) is the III/IV quotient. Reaction mixture: 1 M sorbitol, 10 mM maleate, pH 6.8 (Tris). The subtrate was always 10 mM pyruvate-malate. Three different phosphate (Pi) concentrations and five different KCl concentrations were used as indicated. State III was initiated by adding 500 μM ADP. Mitochondria 0.5 mg prot·(mL)$^{-1}$. Temp 30 °C, final vol 1.5 mL. Data from 3 different experiments.

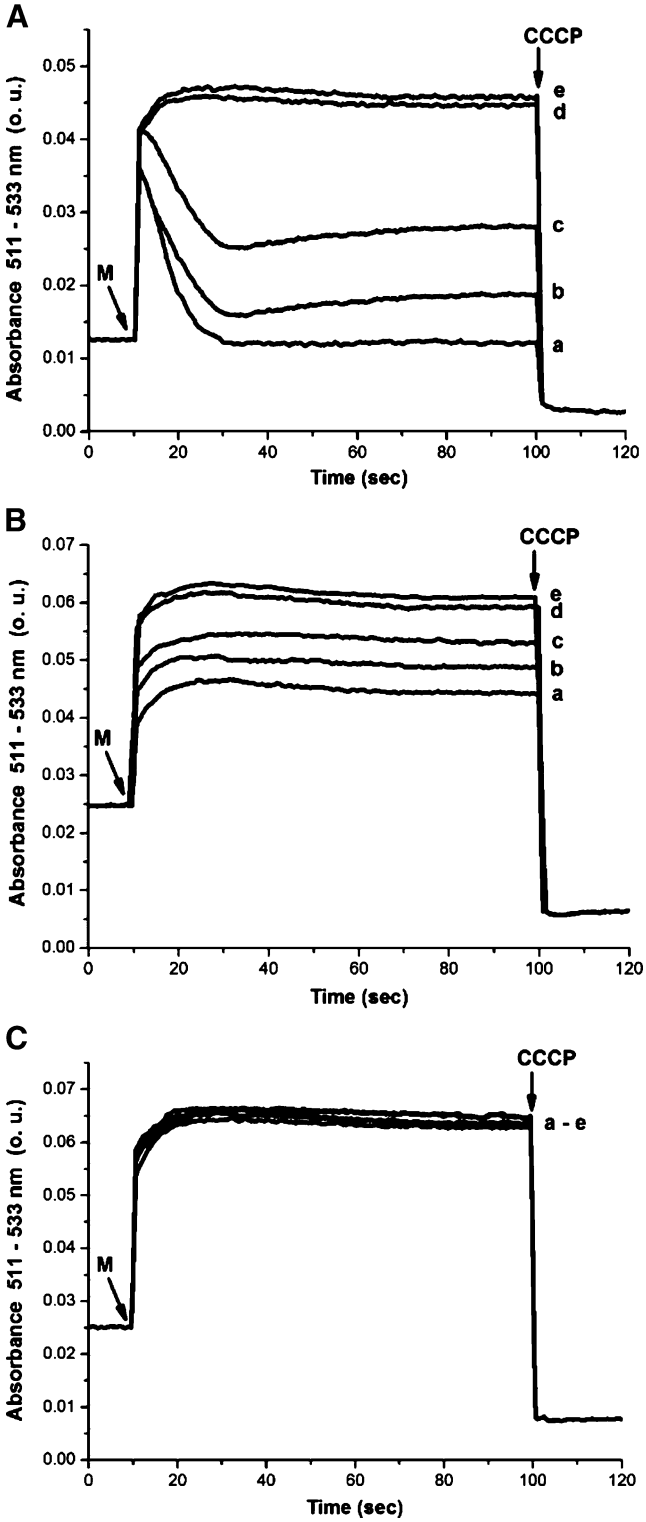

Fig. 1 Effect of K^+ and phosphate on the transmembrane potential ($\Delta\Psi$) of isolated mitochondria from *D. hansenii.* Reaction mixture as in Table 1 except 10 μM safranine-O was added. Final vol. 2 mL; room temperature. Pi concentrations were **A** 0.4 mM; **B** 4.0 mM and **C** 10.0 mM. K^+ concentrations were: **a**, 0; **b**, 10 mM; **c**, 20 mM; **d**, 50 mM; **e**, 75 mM. Where indicated, mitochondria (M) 0.5 mg prot $(ml)^{-1}$ and CCCP 5 μM were added. Representative traces from three independent experiments

The $\Delta\Psi$ sensitivity to monovalent cations does not discriminate between Na^+ or K^+

The possibility that K^+ or Na^+ exhibited different effects was explored by measuring the $\Delta\Psi$ in the presence of three different cation concentrations and in the presence of 0.4 mM Pi. In the absence of cations (Fig. 2 trace a), no increase in $\Delta\Psi$ was observed. In the presence of 18.75 mM Na^+ (Fig. 2 trace b) or K^+ (Fig. 2 trace c), a partial $\Delta\Psi$ was observed. Then, at 37.5 mM monovalent cation, the $\Delta\Psi$ increased to a higher extent regardless of whether the cation was Na^+ (Fig. 2 trace d), K^+ (Fig. 2 trace e), or a mixture of 18.75 mM Na^+ plus 18.75 mM K^+ (Fig. 2 trace f). At 75 mM monovalent cation, a still higher $\Delta\Psi$ was obtained, which again was the same regardless of whether the cation was Na^+ (Fig. 2 trace g), K^+ (Fig. 2 trace h) or a mixture of 37.5 mM Na^+ plus 37.5 K^+ (Fig. 2 trace i). Thus, the opening of the MUC seemed to be equally sensitive to Na^+ or to K^+.

The mitochondrial permeability transition results in decreased synthesis of ATP

In *D. hansenii* mitochondria, PT was triggered by lowering Pi or K^+ and resulted in lower CR and decreased $\Delta\Psi$, leading us to propose that oxidative phosphorylation might be optimized in the presence of increasing concentrations of Pi and K^+. When we tested the synthesis of ATP at three different Pi concentrations and in the presence of different concentrations of K^+, we observed that the rate of synthesis

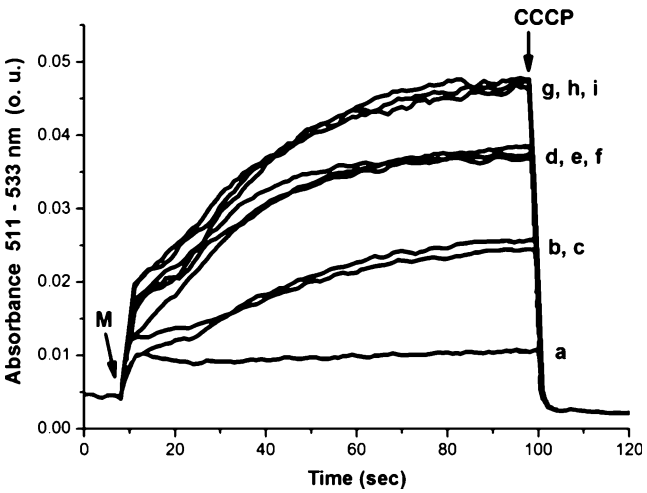

Fig. 2 Effect of Na^+ and K^+ mixtures on the transmembrane potential ($\Delta\Psi$) of isolated mitochondria from *D. hansenii.* Reaction mixture as in Fig. 1. Final volume 2 mL; room temperature. 0.4 mM Pi. Na^+ and/or K^+ concentrations were: **a**, 0; **b**, 18.75 mM Na^+; **c**, 18.75 mM K^+; **d,** 37.5 mM Na^+;18.75 mM Na^+ plus 18.75 mM K^+; **e**, **f**, 37.5 mM K^+; **g**, 37.5 mM Na^+ plus 37.5 mM K^+; **h**, 75 mM Na^+; **i**, 75 mM K^+. Where indicated, mitochondria (M) 0.5 mg prot $(ml)^{-1}$ and CCCP 5 μM were added. Representative traces from three independent experiments

of ATP increased proportionally to Pi and/or K^+, i.e. at 0.4 mM Pi a slow rate of ATP synthesis was observed which increased as the K^+ concentration was raised (Fig. 3). At 4 mM Pi higher rates of ATP synthesis were obtained and a slight optimization of this rate by K^+ was still observed (Fig 3). At the highest concentration of Pi tested, a high rate of synthesis of ATP was observed which was not further increased by the presence of different concentrations of K^+. Thus, the effect on the synthesis of ATP provide further support to the notion that there is an unspecific channel in *D. hansenii* mitochondria which is closed by high concentrations of Pi and by intermediate concentrations of monovalent cations.

The *D. hansenii* MUC is regulated by Ca^{2+} and by Mg^{2+}

Divalent cations modulate the opening of the MUC from different species. However, the effects are opposite as in mammalians Ca^{2+} opens the MUC (Halestrap and Davidson 1990) while in *S. cerevisiae* Ca^{2+} closes the MUC (Pérez-Vázquez et al. 2003) probably acting at the level of the porine (Gutiérrez-Aguilar et al. 2007). By contrast, Mg^{2+} seems to close all known MUCs (Bernardi 1999; Kowaltowski et al. 1998; Pérez-Vázquez et al. 2003). Thus, we decided to characterize the effects of Ca^{2+} and Mg^{2+} on the $\Delta\Psi$ in the presence of 0.4 mM Pi. In the absence of cations (Fig. 4a trace a; Fig. 4b trace a), a low $\Delta\Psi$ was observed. In the presence of increasing Ca^{2+} concentrations, $\Delta\Psi$ increased (Fig. 4a traces a-f); reaching a maximum at 1 mM Ca^{+2}. [Ca^{+2}] above 1 mM did not promote further changes in $\Delta\Psi$ (data not shown). In regard

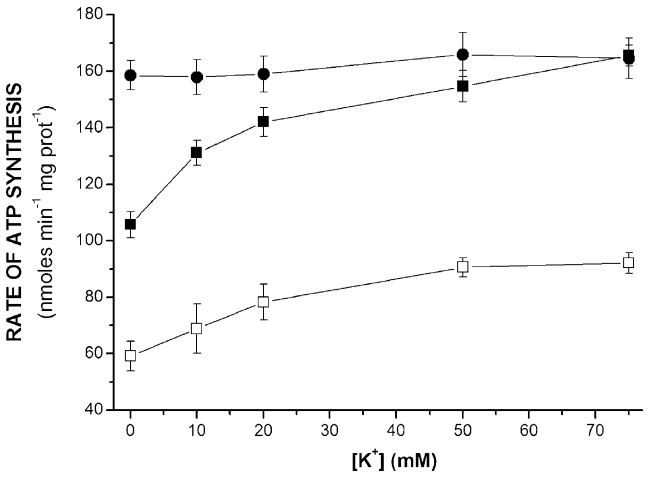

Fig. 3 Effect of K^+ and phosphate on the rate of ATP synthesis mediated by isolated mitochondria from *D. hansenii*. Experimental conditions as in Table 1, 200 μM ADP. In addition, an enzyme coupled ATP assay system was included: 200 μM $MgCl_2$, 20 mM glucose, 1.4 mM $NADP^+$, hexokinase 162.5 μg/mL and glucose-6-phosphate dehydrogenase 2 U/mL. Pi concentrations were: 0.4 mM (□), 4.0 mM (■) and 10.0 mM (●). The reaction was initiated with 200 μM ADP. Each point is the mean of four independent experiments ± SD

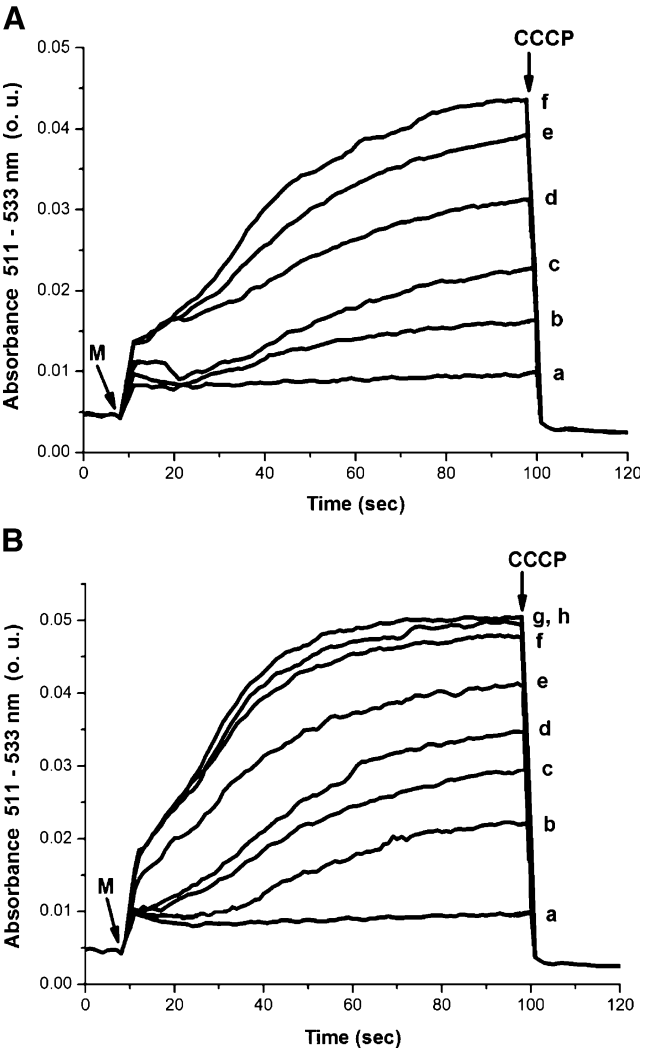

Fig. 4 Effect of divalent cations on the transmembrane potential ($\Delta\Psi$) of isolated mitochondria from *D. hansenii*. Reaction mixture as in Fig. 2, Pi concentration was 0.4 mM. A: Different Ca^{2+} concentrations were: a, 0; b, 100 μM Ca^{2+}; c, 200 μM Ca^{2+}; d, 500 μM Ca^{2+}; e, 750 μM Ca^{2+}; f, 1 mM Ca^{2+}. B: Different Mg^{2+} concentrations were: a, 0; b, 100 μM Mg^{2+}; c, 200 μM Mg^{2+}; d, 500 μM Mg^{2+}; e, 1 mM Mg^{2+}; f, 2 mM Mg^{2+}; g, 5 mM Mg^{2+}; h, 10 mM Mg^{2+}. Where indicated, mitochondria (M) 0.5 mg prot $(ml)^{-1}$ and CCCP 5 μM. Representative traces from three independent experiments

to Mg^{2+}, 100 μM and 200 μM resulted in a partial increase in $\Delta\Psi$ (Fig. 4b, traces a,b). At Mg^{2+} 500 μM and 1 mM, $\Delta\Psi$ was increased to a value near the maximum obtainable (Fig. 4b, traces d,e). At and above 2 mM Mg^{2+} the maximum $\Delta\Psi$ was obtained (Fig. 4b, traces f,g,h). These results suggest that both divalent cations closed the MUC from *D. hansenii*, but this closure was promoted at lower [Ca^{2+}] than [Mg^{2+}].

Selective inhibition of each oxidase and its effect on PT

In *D. hansenii* mitochondria there are two terminal oxidases. In addition to cytochrome oxidase (COX), there

is an alternative oxidase (AOX) (Veiga et al. 2003). The presence of AOX allowed us to explore whether PT might depend on whether electrons were accepted by COX or AOX. Oxygen consumption experiments were conducted in the presence of cyanide (to block COX) or propyl-gallate (to block AOX) (Table 2). The rate of oxygen consumption was measured in state IV and in state III. In the presence of cyanide the rate of oxygen consumption was about one third of the non-inhibited rate (shown in Table 1), although increasing concentrations of K^+ did promote an increase in the rate of oxygen consumption both in state IV and state III, such that the CR remained low (Table 2). This effect suggests that under our conditions AOX expression was low and thus the lack of proton pumping activity by the blocked complexes III and IV failed to establish a high protonmotive force. By contrast, when AOX was blocked, the effect of K^+ on the rate of oxygen consumption did lead to an increase in RC (Table 1) to a similar extent as the increase obtained in the absence of propyl-gallate (see Table 1), indicating that in the absence of the (low) AOX activity PT still occurred.

The possible physiological meaning of the unusual sensitivity to monovalent cations observed in the *D. hansenii* MUC

The monovalent cation-mediated prevention of opening of the *D. hansenii* MUC has not been detected in mitochondria from any other species studied so far. It had always been considered that the permeability to these ions had to be closely controlled in order to avoid a recycling of the cation following a uniport mechanism for uptake and a H^+ antiport mechanism for export that would result in depletion of the electrical gradient and the consequent mitochondrial uncoupling and loss of ATP synthesis (Garlid 1988; Castrejón et al. 1997). This monovalent cation-mediated coupling makes sense when it is considered that *in situ*, *D. hansenii* mitochondria are exposed to high K^+ or Na^+ concentrations. It should be noted that the monovalent cation-promoted increase in the efficiency of the oxidative phosphorylation by *D. hansenii* mitochondria may explain, at least partially the increase in the growth rate of the *D. hansenii* cells when these are exposed to high concentrations of monovalent cations (González-Hernández et al. 2004; Sánchez et al. 2008).

Table 2 Effect of cyanide and propyl-gallate on the rate of oxygen consumption and respiratory control of isolated mitochondria from *Debaryomyces hansenii*

	Cyanide			Propyl Gallate		
$[K^+]$ (mM)	IV	III	RC	IV	III	RC
0	18±0.7	19±0.8	1.02	145±9	162± 11	1.12
10	24.7±0.4	26.8± 1.2	1.08	151± 5	193± 10	1.27
20	25.5±0.3	26.8±0.3	1.05	136±3	203±4	1.50
50	28.6± 1.7	29.7± 2	1.04	144±5	221 ± 7	1.53
75	34±6	36±8	1.06	138±8	240± 13	1.73

Experimental conditions and data as in Table 1 except 100 μM Cyanide or 25 μM propyl gallate were indicated. Data from 4–5 independent experiments.

As more mitochondria are isolated from different species, it is becoming obvious that these organelles are evolving together with the cell to adapt to the environment. As a result, there are variations in the protein composition of the organelle, e.g. the proteins in the respiratory chain vary widely specially in species with branched respiratory chains. It is noteworthy that the components of the respiratory chain do not seem to vary at random, e.g., from 21 yeast species analyzed, 12 contain both an alternative oxidase and complex I (Veiga et al. 2003), while there is only one species, *Pichia anomala*, that contains an alternative oxidase but no complex I (Nosek and Fukuhara 1994).

The effectors regulating the activity of the mitochondrial unspecific channels from different species may vary widely. In many cases, a given molecule has opposite effects in MUCs from different species (Manon and Guérin 1998). This could be an indication of the different functions the MUC might have depending on the species. The mammalian MUC, widely known as the permeability transition pore (PTP) and the *S. cerevisiae* MUC (YMUC) are the best characterized systems. Both channels have a molecule cutoff size of 1.5 kDa (Zoratti and Szabó 1995; Jung et al. 1997) and some cations such as Mg^{2+} and alkylamines close both MUCs (Chávez et al. 2000; Castrejón et al. 2002; Pérez-Vázquez et al. 2003). Here we demonstrated that the *D. hansenii* MUC is also closed by Mg^{2+}. Differences in sensitivity for MUCs from different species to cyclosporin A (CsA) have been reported (Tanveer et al. 1996; Jung et al. 1997). CsA is a potent PT inhibitor in mammalian (Halestrap and Davidson 1990), potato (Fortes et al. 2001) and wheat mitochondria (Pavlovskaya et al. 2007) but has no effect on yeast (Jung et al. 1997) or brine shrimp mitochondria (Menze et al. 2005). In the mammalian PTP, Pi is needed for Ca^{2+}-mediated opening, but it is also required by CsA to inhibit opening (Halestrap and Davidson 1990). In mitochondria from the yeast species *S. cerevisiae* (Velours et al. 1977; Roucou et al. 1997) and *D. hansenii* (this work), Pi always inhibits PT. Ca^{2+} opens the MUC in mammals (Crompton et al. 1988; Bernardi et al. 1994), potato (Fortes et al. 2001) and *N. crassa* (Brustovetsky et al. 2002) but it is inhibitory in *S. cerevisiae* (Jung et al. 1997) and in *D. hansenii* (this work). A Ca^{2+}-porin interaction site has been located both in *S. cerevisiae* (Gutiérrez-Aguilar et al. 2007) and mammals (Israelson et al. 2007). The inability of Ca^{2+} to

open the *S. cerevisiae* MUC might be explained if it is considered that there is no specific carrier for Ca^{2+} in these mitochondria and thus the Ca^{2+} uptake is slow (Uribe et al. 1992). Furthermore, addition of a Ca^{2+} ionophore to *S. cerevisiae* mitochondria enables Ca^{2+} to induce PT (Jung et al. 1997). It would be interesting to analyze the Ca^{2+} transport activity of the isolated mitochondria from *D. hansenii*. Adding to the wide variability in the pattern of PT in mitochondria from different species, recently it was reported that the yeast species *Yarrowia lipolytica* (Deryabina et al. 2004) and *Endomyces magnusii* (Kovaleva et al. 2009) fail to undergo PT unless a high concentration of Ca^{2+} plus the Ca^{2+} ionophore ETH129 are added. Remarkably, *D. hansenii* and *Y. lipolytica* are closely related (Dujon et al. 2004), but the first undergoes PT while the second does so only under very specific conditions (Kovaleva et al. 2009). A comparison between the proteins that have been proposed to constitute the channel might yield abundant information on the mechanisms that lead to PT and on the control mechanisms.

Acknowledgements The authors thank Martha Calahorra, Sergio Couoh, Ramón Méndez, Rocío Romualdo and Norma S. Sánchez for technical assistance. Partially funded by grants from: CONACYT-79989 and UNAM/DGAPA-PAPIIT-IN217109-3. ACO is recipient of a CONACYT undergraduate fellowship 102803.

References

Akerman KEO, Wikström MK (1976) FEBS Lett 68:191–197
Alba-Lois L, Segal C, Rodarte B, Valdés-López V, De Luna A, Cárdenas R (2004) Curr Microbiol 48:68–72
Averet N, Fitton V, Bunoust O, Rigoulet M, Guerin B (1998) Mol Cell Biochem 184:67–79
Bernardi P (1999) Physiol Rev 79:1127–1155
Bernardi P, Broekemeier KM, Pfeiffer DR (1994) J Bioenerg Biomembr 26:509–517
Breuer U, Harms H (2006) Yeast 23:415–437
Brustovetsky N, Tropschug M, Heimpel S, Heidkämper D, Klingenberg M (2002) Biochemistry 41:11804–11811
Castrejón V, Parra MC, Moreno R, Peña A, Uribe S (1997) Arch Biochem Biophys 346:37–44
Castrejón V, Peña A, Uribe S (2002) J Bioenerg Biomemb 34:299–306
Chávez E, Peña A, Zazueta C, Ramírez J, García N, Carrillo R (2000) J Bioenerg Biomembr 32:193–198
Cortés P, Castrejón V, Sampedro JG, Uribe S (2000) Biochim Biophys Acta 1456:67–76
Crompton M, Ellinger H, Costi A (1988) Biochem J 255:357–360
Deryabina YI, Isakova EP, Shurubor EI, Zvyagilskaya RA (2004) Biochemistry (Mosc) 69:1025–1033
Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Marck C, Neuvéglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich JM, Beyne E, Bleykasten C, Boisramé A, Boyer J, Cattolico L, Confanioleri F, De Daruvar A, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud JM, Nikolski M, Oztas S, Ozier-Kalogeropoulos O, Pellenz S, Potier S, Richard GF, Straub ML, Suleau A, Swennen D, Tekaia F, Wésolowski-Louvel M, Westhof E, Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, Souciet JL (2004) Nature 430:35–44
Fadda ME, Mossa V, Pisano MB, Deplano M, Cosentino S (2004) Int J Food Microbiol 95:51–59
Fortes F, Castilho RF, Catisti R, Carnieri EG, Vercesi AE (2001) J Bioenerg Biomembr 33:43–51
Garlid KD (1988) In: Lemasters JJ, Hackenbrock CR, Thurman RG, Westerhoff HV (eds) Integration of Mitochondrial Function. Mitochondrial volume control. Plenum Publishing Corp, New York, pp 259–278
Gómez-Puyou A, Sandoval F, Peña A, Chávez E, Tuena M (1969) J Biol Chem 244:5339–5345
Gómez-Puyou A, Tuena de Gómez Puyou M (1977) J Bioenerg Biomemb 9:91–102
González-Hernández JC, Cárdenas-Monroy CA, Peña A (2004) Yeast 21:403–412
Gornal AG, Bardavill CJ, David MM (1949) J Biol Chem 177:751–760
Guérin B, Bunoust O, Rouqueys V, Rigoulet M (1994) J Biol Chem 269:25406–25410
Gutiérrez-Aguilar M, Pérez-Vázquez V, Bunoust O, Manon S, Rigoulet M, Uribe S (2007) Biochim Biophys Acta 1767:1245–1251
Gutiérrez-Aguilar M, Pérez-Martínez X, Chávez E, Uribe S (2010) Arch Biochem Biophys (in press)
Halestrap AP, Davidson AM (1990) Biochem J 268:153–160
Hobot JA, Jennings DH (1981) Exp Mycol 5:217–228
Israelson A, Abu-Hamad S, Zaid H, Nahon E, Shoshan-Barmatz V (2007) Cell Calcium 41:235–244
Jung DW, Bradshaw P, Pfeiffer DR (1997) J Biol Chem 272:21104–21112
Kovaleva MV, Sukhanova EI, Trendeleva TA, Zyl'kova MV, Ural'skaya LA, Popova KM, Saris NE, Zvyagilskaya RA (2009) J Bioenerg Biomembr 41:239–249
Kowaltowski AJ, Naia-Da-Silva ZS, Castilho RF, Vercesi AE (1998) Arch Biophys Biochem 359:77–81
Kusano T, Tateda C, Berberich T, Takahashi Y (2009) Plant Cell Rep 28(9):1301–1308
Leung AW, Halestrap AP (2008) Biochim Biophys Acta 1777:946–952
Manon S, Guerin M (1997) Biochem Biophys Acta 1318:317–321
Manon S, Guerin M (1998) Biochem Mol Biol Int 44:565–575
Manon S, Roucou X, Guerin M, Rigoulet M, Guerin B (1998) J Bioenerg Biomembr 30:419–429
Menze MA, Hutchinson K, Laborde SM, Hand SC (2005) Am J Physiol Regul Integr Comp Physiol 289:R68–R76
Montiel V, Ramos J (2007) FEMS Yeast Res 7:102–109
Neves ML, Oliveira RP, Lucas CM (1997) Microbiol 143:1133–1139
Nicholls DG, Ferguson SJ (2001). In Bioenergetics 3. Chapter IV, The chemiosmotic proton circuit. Academic Press, pp. 57–87
Norkrans B (1966) Mikrobiol 54:374–392
Norkrans B, Kylin A (1969) J Bacteriol 100:836–845
Nosek J, Fukuhara H (1994) J Bacteriol 176:5622–5630
Pavlovskaya NS, Savinova OV, Grabel'nykh OI, Pobezhimova TP, Koroleva NA, Voinikov VK (2007) Dokl Biol Sci 417:446–448
Peña A, Campillo Serrano C, de Gómez T, Puyou M (1964) Arch Biochem Biophys 106:461–466
Peña MZ, Piña E, Escamilla E, Piña A (1977) FEBS Lett 80:209–213
Pérez-Vázquez V, Saavedra-Molina A, Uribe S (2003) J Bioenerg Biomembr 35:231–241
Prista C, Almagro A, Loureiro-Dias MC, Ramos J (1997) Appl Environ Microbiol 63:4005–4009
Prista C, Loureiro-Dias MC, Montiel V, Garcia R, Ramos J (2005) FEMS Yeast Res 5:693–701

Ramos J (2006) In: Gunde-Cimerman N, Oren A, Plemenitas A (eds) Introducing *Debaryomyces hansenii*, a salt loving yeast. Adaptation to life at high salt concentrations in archaea, bacteria and eukarya. Springer, Berlin, pp 443–451
Roucou X, Manon S, Guerin M (1997) Biochem Mol Biol Int 43:53–61
Sánchez NS, Arreguín R, Calahorra M, Peña A (2008) FEMS Yeast Res 8:1303–1312
Sherman D, Durrens P, Beyne E, Nikolski MJ-L (2004) Nucleic Acids Res 32:D315–D318
Tanveer A, Virji S, Andreeva L, Totty NF, Hsuan JJ, Ward JM, Crompton M (1996) Eur J Biochem 238:166–172
Thomé-Ortiz P, Peña A, Ramírez J (1998) Yeast 14:1355–1371
Uribe S, Ramírez J, Peña A (1985) J Bacteriol 161:1195–1200
Uribe S, Sánchez N, Peña A (1991) Biochem Int 24:615–624
Uribe S, Rangel P, Pardo JP (1992) Cell Calcium 13:211–217
Veiga A, Arabaca JD, Loureiro-Dias MC (2003) FEMS Yeast Res 3:239–245
Velours J, Rigoulet M, Guerin B (1977) FEBS Lett 81:18–22
Zoratti M, Szabo I (1995) Biochim Biophys Acta 1241:139–176