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In *Saccharomyces cerevisiae*, the phosphate carrier is a component of the mitochondrial unselective channel

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ABSTRACT

The mitochondrial permeability transition (PT) involves the opening of a mitochondrial unselective channel (MUC) resulting in membrane depolarization and increased permeability to ions. PT has been observed in many, but not all eukaryotic species. In some species, PT has been linked to cell death, although other functions, such as matrix ion detoxification or regulation of the rate of oxygen consumption have been considered. The identification of the proteins constituting MUC would help understand the biochemistry and physiology of this channel. It has been suggested that the mitochondrial phosphate carrier is a structural component of MUC and we decided to test this in yeast mitochondria. Mersalyl inhibits the phosphate carrier and it has been reported that it also triggers PT. Mersalyl induced opening of the decavanadate-sensitive Yeast Mitochondrial Unselective Channel (YMUC). In isolated yeast mitochondria from a phosphate carrier-null strain the sensitivity to both phosphate and mersalyl was lost, although the permeability transition was still evoked by ATP in a decavanadate-sensitive fashion. Polyethylene glycol (PEG)-induced mitochondrial contraction results indicated that in mitochondria lacking the phosphate carrier the YMUC is smaller: complete contraction for mitochondria from the wild type and the mutant strains was achieved with 1.45 and 1.1 kDa PEGs, respectively. Also, as expected for a smaller channel titration with 1.1 kDa PEG evidenced a higher sensitivity in mitochondria from the mutant strain. The above data suggest that the phosphate carrier is the phosphate sensor in YMUC and contributes to the structure of this channel.

Introduction

In different eukaryotic species a sudden increase in the permeability of the mitochondrial inner membrane to ions and metabolites may occur as a result of the opening of a mitochondrial unspecific channel (MUC)[1] [1–3]. In different species, the mitochondrial permeability transition (PT) may be triggered by different stimuli such as: ischemia in mammals; a high ATP/ADP quotient in *Saccharomyces cerevisiae* or the presence of a pathogenic agent in plants [4–7]. In order to advance in the understanding of the physiological function of PT and its control mechanisms, it is important to determine the structure of the channel in each species under study. To date many data are missing, e.g. it is not clear whether MUCs span only the inner membrane or traverse both membranes or what proteins are involved in each case [1,4,8].

Not all species present a typical PT, as recently evidenced for the yeasts *Yarrowia lypolytica* and *Endomyces magnusii* where a putative PT was evidenced only after the addition of 50–100 μM Ca^{2+} and of the calcium ionophore ETH129 [9,10]. Another species that does not seem to exhibit a typical PT is the invertebrate *Artemia franciscana* where up to 1 mM Ca^{2+} may be added without triggering PT [11]. In all cases, the opening of MUC leads to mitochondrial depolarization and ion-mediated swelling [12–14]. In mammals and plants, PT has been linked to apoptosis and to accidental cell death [8,15,16]. However, in these and other species some alternative roles have been proposed for PT, such as cation detoxification, regulation of ATP synthesis and control of reactive oxygen species (ROS) production [17–20].

In yeast, the physiological significance of PT remains to be established [21]. Several investigators suggest that the yeast mitochondrial PT constitutes an energy surplus escape-valve, e.g. in the stationary growth phase it is necessary to eliminate energy, decreasing anabolism and arresting growth; and both goals would be met through the PT-mediated uncoupling of oxidative phosphorylation

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[1] *Abbreviations used*: PT, mitochondrial permeability transition; MUC, mitochondrial unselective channel; YMUC, yeast mitochondrial unselective channel; ROS, reactive oxygen species; CsA, Cyclosporin A; PTP, permeability transition pore; ANC, adenine-nucleotide carrier; VDAC, voltage-dependent anion channel; PiC, phosphate carrier; TEA, triethanolamine; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; BSA, bovine serum albumin; dVO_4, decavanadate; PAO, phenylarsine oxide; AGC, aspartate/glutamate carrier.

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[22–24]. In addition, uncoupling would increase the activity of the respiratory chain which in turn would prevent excessive production of ROS [25].

It is not clear whether MUCs from different species are equivalent or share the same composition. Plant, invertebrate, mammalian and fungi MUCs do share some properties, e.g., mersalyl or arsine open MUCs in plant [26], *S. cerevisiae* [27], invertebrate [11] and mammalian mitochondria [28]. 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 [12,29]. Also, some cations such as Mg^{2+} and alkylamines close both MUCs [30,31]. In addition to the similarities exhibited by various MUCs, there are also many differences: Cyclosporin A (CsA) is a potent PT inhibitor in citrus, potato, wheat and mammalian mitochondria [2,26,32,35] but has no effect on brine shrimp or yeast mitochondria [11,29]. It is noteworthy that in mammalian mitochondria, the absence of phosphate (Pi) [33] or aging [34] render the cyclophylin-D- or the mersalyl-induced PT insensitive to CsA. Pi has a dual effect on the mammalian PTP as it potentiates opening by Ca^{2+}, but is required for CsA-mediated inhibition. In other words, Pi inhibits rather than activates PT when Cyclophylin-D is detached from mitochondria [33]. In the yeast species *Saccharomyces cerevisiae* [5] and *Debaryomyces hansenii* (to be published), Pi always inhibits PT. The adenine-nucleotide carrier (ANC) inhibitor, bongkrekic acid inhibits the mammalian system while it opens the YMUC [35,36]. ATP inhibits the PTP [37] while it opens the YMUC [22].

Ca^{2+} loading triggers PT in mitochondria from mammals [12,17], potato [26] and in the reconstituted ANC from *N. crassa* [38] while it lacks effects in brine shrimp [10] and prevents the PT in *S. cerevisiae* [30] and *D. hansenii* (to be published). The Ca^{2+} effect (positive or negative) is potentiated by Pi. In the presence of ruthenium red, Ca^{2+} inhibits the mammalian PT at an external site probably at the porine, also called the voltage-dependent anion channel (VDAC), which has been identified as an external membrane component of MUC [14,39]. The Ca^{2+}-VDAC interaction site has been located both in yeast [40] and mammalians [8]. Interestingly, a Ca^{2+}-induced permeability transition can be triggered in *S. cerevisiae* using the Ca^{2+} ionophore ETH129, i.e. when Ca^{2+} enters the matrix rapidly [41]. Thus, the slow Ca^{2+} uptake by *S. cerevisiae* mitochondria may explain the resistance to the Ca^{2+}-promoted PT [42,43].

Many studies indicate that in the internal mitochondrial membrane, the MUC channel is constituted by ANC [35]. However, recent evidence points to the phosphate carrier (PiC) as the structural component of the pore [44]. In addition, it has been suggested that both the ANC and the PiC might associate to form the channel [45]. These carriers seem to interact forming a large molecular complex containing also the F_1F_0 ATP synthase and VDAC [46]. In this structure, termed the synthasome, the association of PiC and ANC to form a channel would probably be facilitated. The relationship with VDAC is more complicated since it has been shown that this protein is dispensable for PTP and YMUC opening, which raises the possibility that this protein is a regulatory component of MUC [40,47].

It was decided to determine whether PiC may be part of the YMUC. Our approach was to analyze the mersalyl-promoted PT in isolated *S. cerevisiae* mitochondria from a wild type strain and from a strain where PiC had been deleted (*ΔPiC*). In order to ensure that the mersalyl-mediated effect occurred at YMUC and not just at PiC, we tested the inhibitory effect of decavanadate (dVO_4). As dVO_4 closes the YMUC by interacting with another putative YMUC protein (VDAC), inhibition of the mersalyl effect would strongly suggest that these effects occur at the VDAC/PiC-containing YMUC and not just at PiC [40], i.e. isolated WT mitochondria were sensitive to mersalyl in a dVO_4 sensitive fashion, indicating that there is an interaction between VDAC and PiC. Furthermore, polyethylene glycol (PEG)-induced contraction experiments suggested that YMUC had a smaller diameter in the *ΔPiC* strain. Our results suggest that the phosphate carrier is a component of the YMUC.

Material and methods

Materials

All chemicals were reagent grade. Mannitol, MES, triethanolamine (TEA), orthovanadate, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), safranine-O, KCl, mersalyl and bovine serum albumin (BSA) type V were purchased from Sigma Chem. Co. (St. Louis, MO). All other reagents were of the highest purity available. Decavanadate (dVO_4) was obtained as in [36]. The *Saccharomyces cerevisiae* strains were a gift from Dr. Stephen Manon (Institut de Biochimie et Génétique Cellulaires, Université de Bordeaux-2 Victor Segalen, Bordeaux, France).

Yeast strains and growth conditions

The yeast strains BY4741 *MATa; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ* (WT) and BY4741 *MATa; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0*; YJR077c: kanMX4 (*ΔPiC*) were used for the isolation of yeast mitochondria. Both strains were a kind gift from Dr. Stephen Manon, Institut de Biochimie et Génétique Cellulaires, Université de Bordeaux-2, Victor Segalen, France. Yeast cells were incubated in YPD preculture medium. for 24 h at 30 °C, under agitation at 250 rpm YPD was 1% yeast extract, 2% peptone, 0.1% potassium phosphate, 0.12% ammonium sulfate, pH 5.5, plus 2% glucose. Subsequently cultured in YPlac (1% yeast extract, 2% peptone, 1% KH_2PO_4, 12% $(NH_4)_2SO_4$, 2% lactic acid, pH 5.0) or YPgal medium (1% yeast extract, 2% peptone, 0.1% potassium phosphate, 0.12% ammonium sulfate, pH 5.5, plus 2% galactose) at 250 rpm, 30 °C for approximately 24 h until they reached an optical density of 3–3.5 at 600 nm.

Isolation of mitochondria

Mitochondria were obtained as described by Guerin et al. [48]. Cells were washed and resuspended in preincubation buffer for 15 min (0.1 M Tris, 0.5 M β-mercaptoethanol, pH 9.3). In order to remove excess β-mercaptoethanol, the cells were washed 3 times with KCl buffer (0.5 M KCl, 10 mM Tris pH 7.0) and resuspended in digestion buffer (1.35 M sorbitol, 1 mM EGTA, 10 mM citric acid, 10 mM NaH_2PO4, 10 mM Na_2HPO4, pH 5.8), and zymoliase was added at 10 mg/g cell dry weight. Spheroplast formation was followed measuring cell fragility in water at 600 nm in a Beckman DU-50 spectrophotometer [49]. Spheroplasts were washed in spheroplast buffer (0.75 M sorbitol, 0.4 M mannitol, 10 mM Tris, 0.1% BSA, pH 6.8), resuspended in homogenization buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris, 0.2% BSA, pH 6.8) and disrupted with 20 passes in a Potter–Evelheim homogenizer equipped with a tight pestle. Then, mitochondria were isolated by differential centrifugation in a SS34 Sorvall rotor [48]. Protein concentration was determined by the biuret method [50].

Respiration assay

The rate of oxygen consumption was measured in the resting state (State 4) and in the presence of the uncoupler FCCP (State U), using a YSI model 5300 Oxygraph equipped with a Clark electrode [51] in a 3 mL water jacketed chamber (at 30 °C). Mitochondria (0.5 mg protein/mL) were added to a reaction mixture containing 0.6 M mannitol, 5 mM MES, pH 6.8 (TEA), 20 mM KCl.