Biochemistry (Moscow), Vol. 69, No. 9, 2004, pp. 1025-1033. Translated from Biokhimiya, Vol. 69, No. 9, 2004, pp. 1261-1270.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-034, August 29, 2004.

Calcium-Dependent Nonspecific Permeability of the Inner Mitochondrial Membrane Is Not Induced in Mitochondria of the Yeast *Endomyces magnusii*

Y. I. Deryabina, E. P. Isakova, E. I. Shurubor, and R. A. Zvyagilskaya*

Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, Moscow 119071, Russia; fax: (7-095) 954-2732; E-mail: renata_z@inbi.ras.ru

Received February 12, 2004
Revision received March 30, 2004

Abstract—Mitochondria of the yeast *Endomyces magnusii* were examined for the presence of a Ca^{2+}- and phosphate-induced permeability of the inner mitochondrial membrane (pore). For this purpose, coupled mitochondria were incubated under conditions known to induce the permeability transition pore in animal mitochondria, i.e., in the presence of high concentrations of Ca^{2+} and P_i, prooxidants (*t*-butylhydroperoxide), oxaloacetate, atractyloside (an inhibitor of ADP/ATP translocator), SH-reagents, by depletion of adenine nucleotide pools, and deenergization of the mitochondria. Large amplitude swelling, collapse of the membrane potential, and efflux of the accumulated Ca^{2+} were used as parameters for demonstrating pore induction. *E. magnusii* mitochondria were highly resistant to the above-mentioned substances. Deenergization of mitochondria or depletion of adenine nucleotide pools have no effect on low-amplitude swelling or the other parameters. Cyclosporin A, a specific inhibitor of the nonspecific permeability transition in animal mitochondria, did not affect the parameters measured. It is thus evident that *E. magnusii* mitochondria lack a functional Ca^{2+}-dependent pore, or possess a pore differently regulated as compared to that of mammalian mitochondria.

Key words: mitochondria, *Endomyces magnusii*, membrane potential, Ca^{2+}-transport, Ca^{2+}-induced nonspecific permeability

Calcium plays an important role as an intracellular messenger in signal transduction [1-3]. In animal mitochondria, elevated matrix Ca^{2+} concentrations up to 1-3 μM induce an increased permeability of the inner mitochondrial membrane due to opening of a nonspecific pore (Permeability Transition Pore, PTP) (for reviews see [3, 4]). The Ca^{2+}-dependent pore has been postulated to be a complex structure formed from the adenine nucleotide translocase, the peripheral benzodiazepine receptor, the outer membrane voltage-dependent channel, and cyclophilin D [3]. This complex, following binding of Ca^{2+}, forms a pore 2.6-2.9 nm in diameter that allows solutes of molecular mass ≤1500 daltons to equilibrate across the inner membrane, resulting in a decrease in the membrane potential value, release of the accumulated Ca^{2+}, and high-amplitude swelling of mitochondria [3-6]. Pore opening is stimulated by inorganic phosphate (P_i), prooxidants, SH-reagents, and depletion of the ATP pool. Conversely, Mg^{2+} [7], adenine nucleotides [3, 6, 8], spermine [8], antioxidants, and cyclosporin A (CsA) [9] are potent inhibitors of the pore. The Ca^{2+}-dependent PTP is considered now as playing a role in the crosstalk of two different physiological pathways, one of which takes part in the Ca^{2+}-dependent signal transduction during the cell cycle [6], while the other initiates a reaction cascade leading to apoptosis [3, 6, 10, 11] or necrosis [12] in animal cells.

The presence of a PTP-like pore in yeast mitochondria is still uncertain. Mitochondria from the yeast *Saccharomyces cerevisiae*, unlike animal counterparts, are resistant to relatively high ambient [Ca^{2+}] [13]. This was attributable to the lack in brewer yeast mitochondria of a Ca^{2+} uptake pathway of any significant physiological relevance [14, 15] or to the presence in yeast cells a highly active antioxidant system [13, 16, 17], preventing oxidative stress, and ultimately, PTP induction. Jung et al. [18] found that *S. cerevisiae* mitochondria, when incubated in

Abbreviations: CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; PTP) permeability transition pore; Δψ) mitochondrial inner membrane transmembrane potential; Atr) atractyloside; CsA) cyclosporin A; OA) oxaloacetate; PhAsO) phenylarsine oxide.

* To whom correspondence should be addressed.

0006-2979/04/6909-1025 ©2004 MAIK "Nauka/Interperiodica"

isotonic medium containing ethanol (oxidizable substrate), the Ca^{2+} ionophore ETH129, and 10 mM P_i, accumulated large amounts of Ca^{2+} without classical PTP (pore) induction. However, a Ca^{2+}-independent, CsA-insensitive pore did arise in response to ATP. Kowaltowski and coauthors [13] showed that a Ca^{2+}-dependent PTP in permeabilized *S. cerevisiae* spheroplasts could be induced only by high (non-physiological) [Ca^{2+}] in spheroplasts from wild type cells under oxidative stress conditions (in the presence of SH-reagents and inhibitors of catalase) and from mutants lacking the thioredoxin peroxidase gene. PTP induction was accompanied by enhanced production of hydrogen peroxide and decreased cell viability. At present, it is difficult to construct a comprehensive picture of PTP induction in yeast mitochondria using as a background the above-mentioned conflicting data. It seems reasonable that if *S. cerevisiae* mitochondria could have elevated permeability transition of the inner mitochondrial membrane, the pore would display properties differing significantly from those of the PTP or possess a pore not coupled with Ca^{2+} uptake (*S. cerevisiae* mitochondria have no Ca^{2+}-uptake system) and differently regulated compared to that of animal mitochondria. Additionally, the absence of considerable changes in the volume of *S. cerevisiae* mitochondria may be associated with some peculiarities of their ultrastructure, as they contain few cristae and therefore have a decreased extending and stretching capacity [18]. This means that the maximal amplitude of swelling of yeast mitochondria is expected to be less than that of the animal counterparts.

This study was undertaken to examine mitochondria from the yeast *E. magnusii* harboring the high-capacity, effectively regulated Ca^{2+} uniporter [19-26] and a separately functioning Na^+-independent pathway for Ca^{2+} efflux [27, 28] for the presence of a Ca^{2+}-dependent PTP. Remarkably, *E. magnusii* mitochondria are very similar in their structure with heart mitochondria as they contain a large number of well-structured cristae [29].

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), EDTA, EGTA, sorbitol, mannitol, spermine, murexide, $CaCl_2$, ADP, ATP, Tris, pyruvate, malate, valinomycin, nigericin, atractyloside, dithiothreitol, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma (USA), Coomassie G-250, safranine O, and NADH from Serva (Germany), A23187 from Boehringer Mannheim (Germany), and cyclosporin A (CsA) was a kind gift of Novartis (Switzerland). Other reagents of analytical grade (of highest quality available) were obtained from domestic suppliers.

Organism and growth conditions. The yeast *E. magnusii*, strain VKM Y-261, was grown in semi-synthetic medium with 0.6-1% glycerol as a sole carbon source as described earlier [30]. Cells were harvested at the late exponential growth phase (10 to 13 g wet weight per liter).

Mitochondria were isolated by the method developed in our laboratory and described earlier [26].

Depletion of the intramitochondrial adenine nucleotide pools was performed by supplementing grinding medium and washes with 10 mM pyrophosphate [31].

Deenergization of mitochondria was monitored at 540 nm as recommended by Halestrap [32] after a 1-min incubation in 0.4 M mannitol containing 0.1 M KCl, 20 mM Tris-acetate, pH 7.4, antimycin A (8 μg/mg protein), and 2 μM A23187, with subsequent addition of 100-500 μM Ca^{2+} and pore-inducing agents.

Mitochondrial swelling was monitored with a Hitachi-557 spectrophotometer (Japan) by recording changes in absorbance at 540 nm in 0.4 M mannitol containing 0.1 M KCl, 2 mM Tris-phosphate or 20 mM Tris-acetate, pH 7.4, 20 mM Tris-pyruvate, 5 mM Tris-malate, and mitochondrial protein corresponding to 0.4 mg/ml.

Ca^{2+} uptake was assayed with a Hitachi-557 spectrophotometer using dual wavelength photometry at 507-540 nm with 50-μM murexide as a metallochromic Ca^{2+}-indicator [33].

Mitochondrial transmembrane potential ($\Delta\psi$) was measured at the wavelength pair 511-533 nm with 10-20 μM safranine O [34].

Mitochondrial protein was assayed by the method of Bradford [35] with BSA as standard.

RESULTS

The most important markers of the PTP of the inner mitochondrial membrane are: high-amplitude swelling of mitochondria due to non-controlled entry of osmolytes with M_r of ≤1500 daltons and, ultimately, water; a decrease in the mitochondrial membrane potential ($\Delta\Psi$); and a rapid release of the accumulated Ca^{2+}, all together indicating that mitochondria are becoming loosely coupled because of disturbing of the membrane integrity. To reveal a Ca^{2+}-dependent nonspecific pore in yeast mitochondria, we investigated the swelling of mitochondria in the presence of various [Ca^{2+}] and different PTP-triggering agents including P_i, oxaloacetate (OA), SH-reagents (N-ethylmaleimide, menadione, phenylarsine oxide (PhAsO)), atractyloside (Atr) (an inhibitor of the adenine nucleotides translocase), and the uncoupler CCCP. As the rate of electrogenic K^+ influx was shown [36] to be a decisive factor determining the rate of swelling of animal mitochondria, the yeast incubation medium was supplemented with 0.1 M KCl.

Yeast mitochondria, in contrast to the animal counterparts, did not show high-amplitude swelling even in the

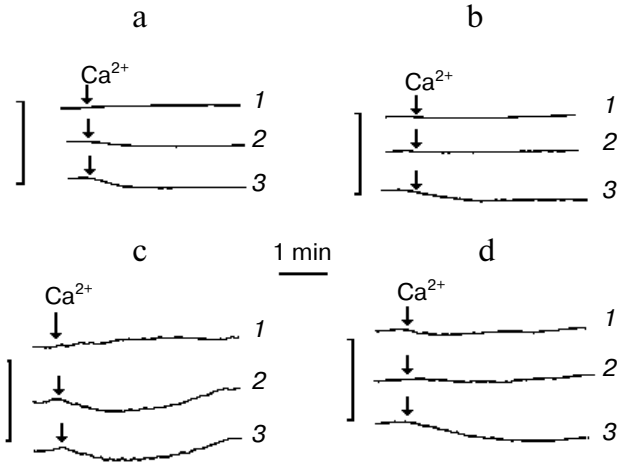

Fig. 1. Ca^{2+}-induced swelling of *E. magnusii* mitochondria in the presence of 100 (*1*), 300 (*2*), and 500 μM Ca^{2+} (*3*) and varying amounts of phosphate (P_i, mM): 2 (a); 5 (b); 7 (c); 10 (d). The incubation medium contained 0.4 M mannitol, 10 mM Tris-phosphate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.5 mg mitochondrial protein. $A = 0.1$ (a, b) and 0.02 (c, d).

presence of massive Ca^{2+} corrected text loads (600-1000 nmol/mg protein) and P_i (Fig. 1a), the most potent inducers of the PTP. Addition of 100 μM Ca^{2+} (Fig. 1a, *1*) induced only a slight increase ($\Delta A = 0.012$-0.014) in the mitochondrial volume. The swelling amplitude increased only insignificantly in the presence of increasing P_i concentrations (Fig. 1, b-d, *1*). Just the opposite, a decrease in the organelle volume was observed. In the control experiments (not shown), increased P_i concentrations (≥2 mM) inhibited CsA-independent Ca^{2+} uptake by yeast mitochondria. It is reasonable to suggest that the observed decrease in the amplitude of mitochondrial swelling in the presence of elevated P_i concentrations resulted from the hampered entry of Ca^{2+} in the matrix space. Higher, 300-500 μM Ca^{2+}, initiating irreversible alterations in mitochondrial functions in animal tissues [8], induced only low-amplitude swelling of yeast mitochondria (Fig. 1, *2* and *3*). The addition of high [Ca^{2+}] did not produce either massive release of the Ca^{2+} accumulated (Fig. 2a) or dramatic decrease in ΔΨ after uptake of the cation added (Fig. 2b).

We did not observe the hallmarks of the classical PTP in yeast mitochondria even in the presence of millimolar [Ca^{2+}]. Figure 3 depicts that in *E. magnusii* mitochondria, 1, 2, and 5 mM Ca^{2+} triggered only low-amplitude swelling ($\Delta A = 0.01$-0.02) (Fig. 3a) and a moderate, reversible decrease in ΔΨ possibly reflecting cation translocation in the mitochondrial matrix (Fig. 3b). Mitochondria took up 1 mM Ca^{2+} with the rate of 650 nmol/min per mg protein, approximately one third of the Ca^{2+} added was accumulated (Fig. 3c, *1*). The uptake rate considerably decreased in the presence of 2 or 5 mM

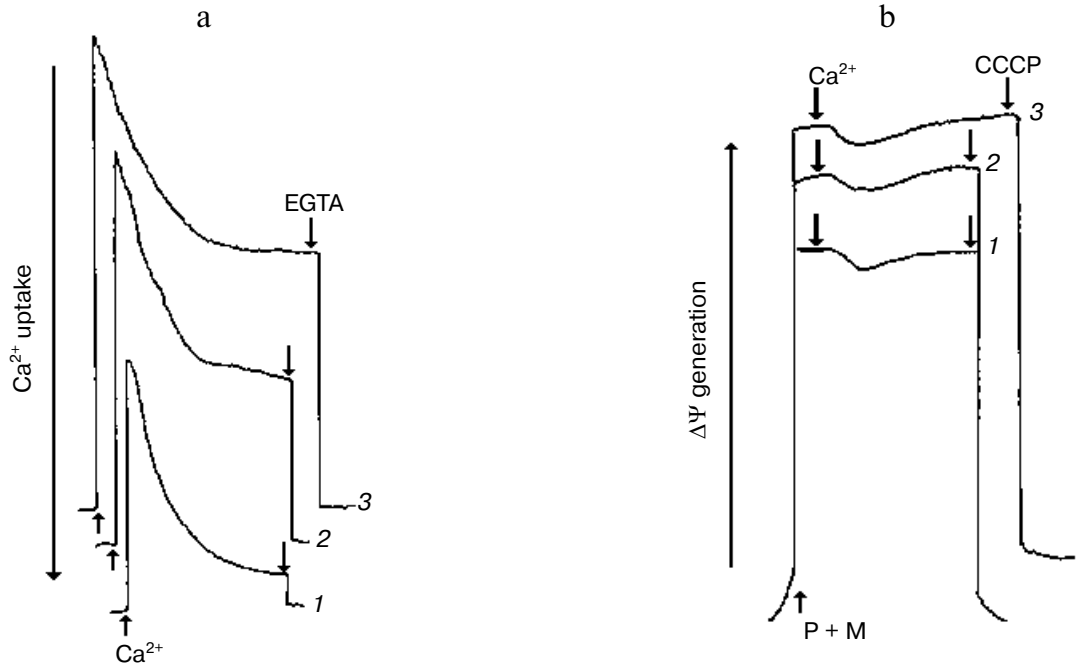

Fig. 2. Photometric recording of Ca^{2+} uptake (a) and Δψ (b) in *E. magnusii* mitochondria. The incubation medium contained 0.4 M mannitol, 10 mM Tris-phosphate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.5 mg mitochondrial protein. Where indicated, 100 (*1*), 300 (*2*), or 500 μM Ca^{2+} (*3*), 1 mM EGTA, or 1 μM CCCP was added. Designations: P + M, pyruvate + malate.

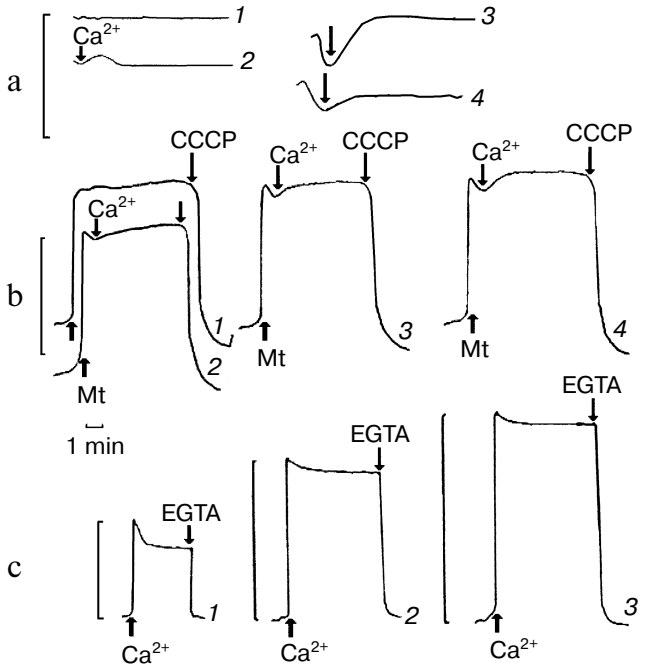

Fig. 3. Photometric recording of swelling (a), $\Delta\psi$ (b), and Ca^{2+} uptake (c) in *E. magnusii* mitochondria. The incubation medium contained 0.4 M mannitol, 10 mM Tris-phosphate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.5 mg mitochondrial protein. a, b: *1*) no Ca^{2+} was added; where indicated, 1 (*2*), 2 (*3*), or 5 mM Ca^{2+} (*4*), or 3 μM CCCP was added. c) 1 (*1*), 2 (*2*), or 5 mM Ca^{2+} (*3*); 2 M (*1*, *2*) or 5 mM EGTA (*3*) was added. A = 0.05 (a) and 0.02 (b), respectively. Designations: Mt, mitochondria.

Ca^{2+} and mitochondria accumulated only 2% of the Ca^{2+} added (Fig. 3c, *2* and *3*), thus indicating inhibition of Ca^{2+}-transporting capacity of yeast mitochondria in the presence of very high, non-physiological [Ca^{2+}]. A 1-min preincubation of mitochondria with CsA did not perturb the Ca^{2+} uptake pattern, the membrane potential value, or changes in the organelle volume (not shown), thus definitely indicating lack of a PTP in yeast mitochondria in the presence of high Ca^{2+} and P_i concentrations.

Because P_i, one of the most potent inducers of PTP in mitochondria of all studied animal tissues, did not induce a pore in yeast mitochondria even in the in the presence of high [Ca^{2+}], our next step was to examine effects of other agents which are known (considered) as triggers of the PTP in animal mitochondria: Atr [8, 37], N-ethylmaleimide [9, 38], menadione [39], PhAsO [13, 40], OA [9], and EGTA + CCCP [41, 42]. To prevent the effect of P_i, all media contained acetate instead of phosphate as permeable anion. In the control experiments, the addition of 100, 300, or 500 μM Ca^{2+} to *E. magnusii* mitochondria initiated predictable, dependent on the ion concentration, changes in the swelling amplitude and a reversible decrease in $\Delta\Psi$ values, but not PTP (Fig. 4). Mitochondria accumulated about 70% of the Ca^{2+} added (Fig. 4c), releasing the accumulated Ca^{2+} due to activation of the Ca^{2+}/nH^+-antiporter of yeast mitochondria, whose properties and regulation have been previously described [27, 28]. Upon examining effects of several agents that influence PTP, some cautions were taken into consideration. Before addition of AO, the medium was supplemented with antimycin A in order to prevent the subsequent mobilization of malate from OA in the malate dehydrogenase reaction [43]. In experiments with the uncoupler CCCP, 0.2 mM EGTA was added 1 min after addition of Ca^{2+} in order to block possible reversible activity of the Ca^{2+}-uniporter thus allowing to maintain high [Ca^{2+}] in the matrix space [44], which is necessary for PTP development [45], and to prevent redistribution of Ca^{2+} between subpopulations of mitochondria with different stability and to inhibit progression of spontaneous PTP [38, 46]. With all these factors considered, in the presence of 100 and 300 μM Ca^{2+}, none of the above mentioned PTP triggers induced high-amplitude swelling of yeast mitochondria (Fig. 5). The effect of each potential PTP inducer on the organelle volume correlated with its action on the Ca^{2+}-transport system (Fig. 6c). EGTA (0.2 mM) partially chelated Ca^{2+} (Fig. 6c, *2*), then [Ca^{2+}]

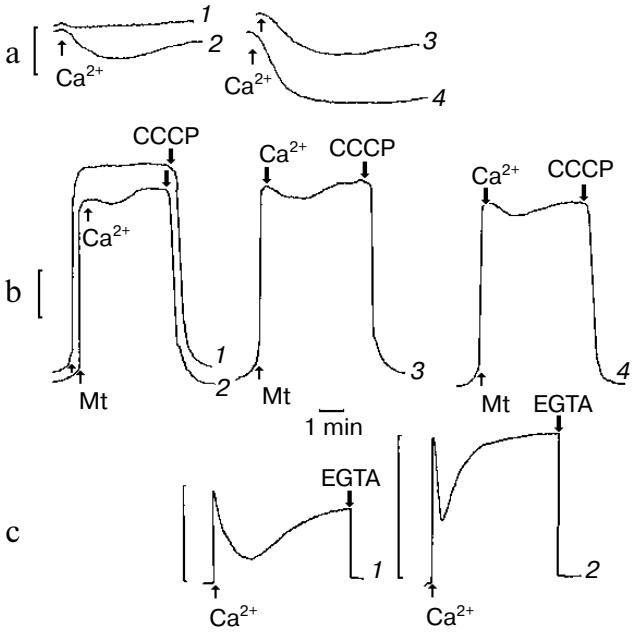

Fig. 4. Photometric recording of swelling (a), $\Delta\psi$ (b), and Ca^{2+} uptake (c) in *E. magnusii* mitochondria suspended in acetate-containing medium. The incubation medium contained 0.4 M mannitol, 20 mM Tris-acetate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.4 mg of mitochondrial protein. a, b: *1*) no Ca^{2+} was added; where indicated, 100 (*2*), 300 (*3*), or 500 μM Ca^{2+} (*4*), or 3 μM CCCP was added. c) 300 (*1*) or 500 μM Ca^{2+} (*2*); 0.3 (*1*) or 0.5 M EGTA (*2*) was added. A = 0.02 (a, b). Designations: Mt, mitochondria.

increased, probably due to release of the accumulated cation. CCCP, being added 1 min after EGTA, did not affect Ca^{2+} release. SH-reagents such as N-ethylmaleimide, PhAsO, and menadione (Fig. 6c, *3*, *6*, and *7*, respectively), being added to the mitochondrial suspension after Ca^{2+}, considerably inhibited swelling of mitochondria by blocking Ca^{2+} accumulation. All these compounds also insignificantly decreased the rate of Ca^{2+} release from mitochondria. OA and Atr (Fig. 6c, *4* and *5*, respectively) did not affect the organelle volume. Atr had no impact on the Ca^{2+} transport process, while OA inhibited Ca^{2+} uptake, most probably decreasing the intramitochondrial NADH/NAD ratio. All these potential PTP triggers changed $\Delta\Psi$ differently (Fig. 6b). CCCP and antimycin A (Fig. 6b, *2* and *4*, respectively) immediately produced the collapse of $\Delta\Psi$ as a result of uncoupling and inhibition of the electron transport in the respiratory chain, respectively. N-Ethylmaleimide and PhAsO (Fig. 6b, *3* and *6*, respectively) brought on a slow decrease in $\Delta\Psi$, possibly as a result of interacting with c SH-groups of membrane proteins. In the control experiments, the same effects of the potential PTP triggers were observed when Ca^{2+} was omitted from the incubation medium, providing addition weight to the conclusion that all these compounds did not induce PTP in yeast mitochondria. Thus, energized *E. magnusii* mitochondria did not display an increase in the membrane permeability in the presence of high Ca^{2+} concentrations and a number of potential PTP inducers.

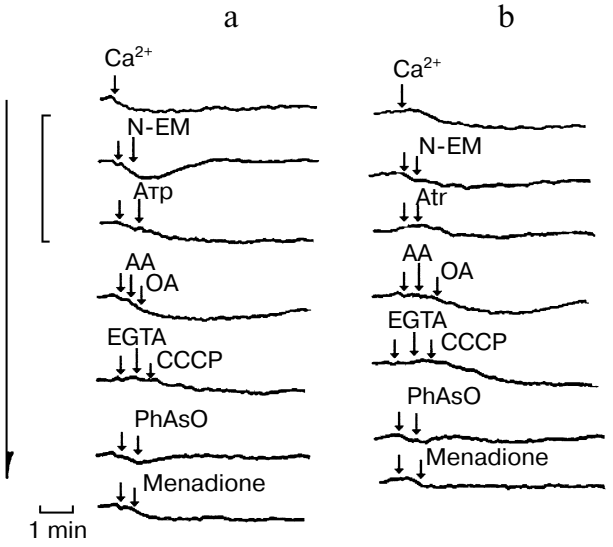

Fig. 5. Photometric recording of swelling of *E. magnusii* mitochondria in the presence of 100 (a) or 300 μM Ca^{2+} (b). The incubation medium contained 0.4 M mannitol, 20 mM Tris-acetate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.4 mg of mitochondrial protein. Where indicated, 500 μM N-ethylmaleimide, 30 μM Atr, 8 μg antimycin A (AA) per mg protein and then 0.1 mM OA, 0.2 mM EGTA and then 1 μM CCCP, 50 μM PhAsO, or 50 μM menadione was added. $A = 0.1$.

As we described above, one of the factors limiting changes in the volume of yeast mitochondria may be ultrastructural organization of their cristae. *E. magnusii* are characterized by well-structured cristae [29], resembling in this respect heart mitochondria, which suggests potentially high amplitude of mitochondrial swelling. In order to diminish this limitation, experiments with pore-forming alamethicin were performed. Antibiotic alamethicin, being inserted into the mitochondrial membrane, forms channels with a diameter of 1 nm that are permeable for divalent cations and low-molecular-weight solutes, resembling in this aspect the Ca^{2+}-dependent PTP [47]. Figure 7 depicts that the amplitude of swelling of yeast mitochondria in the presence of high [Ca^{2+}] and various PTP inducers in media with varying ionic strength (see legend to Fig. 7) was approximately 10-fold less compared to the same parameter after addition of alamethicin ($\Delta A = 0.2$), thus giving additional evidence for the lack of high-amplitude swelling (one of the most important hallmarks of PTP development) of yeast mitochondria.

Previously, it has been demonstrated that deenergization of mitochondria [32] or depletion of the intramitochondrial pools of adenine nucleotides [8, 31, 41] make mitochondria more sensitive to massive Ca^{2+} loads, favoring the PTP development. We examined, on these experimental grounds, effects of potential PTP inducers on deenergized *E. magnusii* mitochondria; deenergization was performed as recommended in [32]. Briefly, the mitochondrial suspension was preincubated for 1 min with antimycin A, an inhibitor of the respiratory chain (to prevent oxidation of endogenous substrates), and with Ca^{2+}-ionophore A23187 (to equilibrate Ca^{2+} between two surfaces of the inner mitochondrial membrane) (for details, see "Materials and Methods"). After a 1-min preincubation, mitochondrial suspension was supplemented with 500 μM Ca^{2+} and potential PTP triggers. However, even under these conditions, we did not observe appreciable mitochondrial swelling ($A = 0.003$-0.007), the swelling amplitude being one order of magnitude less than that in energized yeast mitochondria (Fig. 8a).

Similar results were obtained when the Ca^{2+}-ionophore A23187 was replaced by the uncoupler CCCP (not shown). Low-amplitude swelling of deenergized yeast mitochondria was parallel to their inability to take up Ca^{2+} (Fig. 8b). Adenine nucleotide-depleted mitochondria fail to swell following 500 μM Ca^{2+} addition (Fig. 9). The only unusual feature in the behavior of the mitochondria was the lack of spontaneous Ca^{2+} release of the accumulated cation (Fig. 9c), most probably because of the high (10 mM) pyrophosphate concentration used, which is known to decrease [Ca^{2+}] in the matrix space, thus preventing activation of the Ca^{2+}/nH^{+}-antiporter.

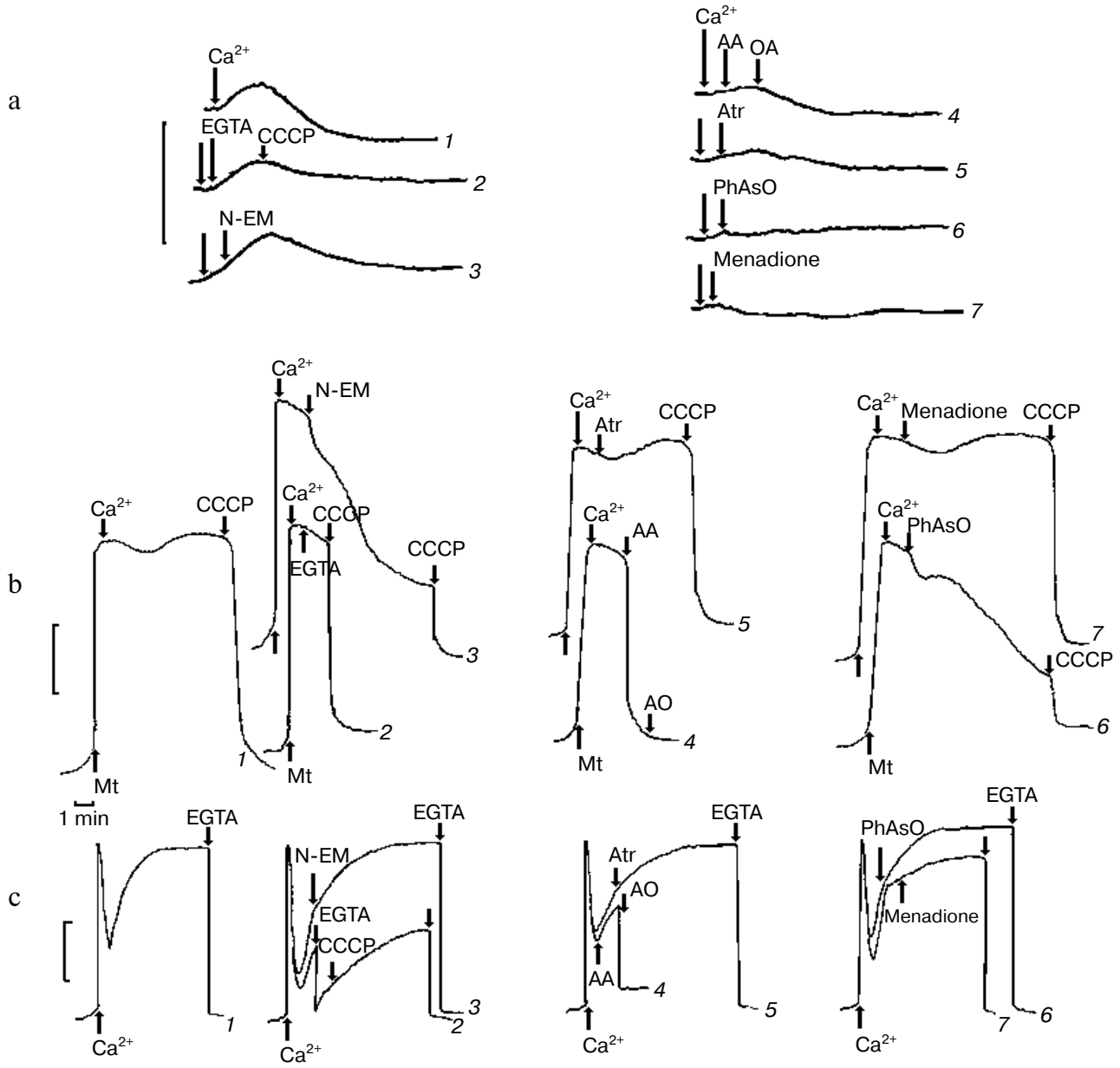

Fig. 6. Photometric recording of swelling (a), $\Delta\psi$ (b), and uptake of 500 μM Ca^{2+} (c) by *E. magnusii* mitochondria. The incubation medium contained 0.4 M mannitol, 20 mM Tris-acetate, pH 7.4, 0.1 M KCl, 20 mM pyruvate, 5 mM malate, and mitochondria corresponding to 0.4 mg of mitochondrial protein. Where indicated (b), mitochondria (Mt) (0.5 mg mitochondrial protein) were added. Where indicated, 0.2 mM EGTA and then 1 μM CCCP, 500 μM N-ethylmaleimide, 8 μg/mg antimycin A and then 0.1 mM OA, 30 μM Atr, 50 μM PhAsO, or 50 μM menadione was added. c) Ca^{2+} uptake reactions were terminated by addition of 0.5 M EGTA. $A = 0.1$ (a) and 0.02 (b), respectively.

DISCUSSION

Thus, tightly-coupled *E. magnusii* mitochondria do not undergo Ca^{2+}-induced permeabilization or opening of a PTP even when treated with millimolar [Ca^{2+}] and agents (P_i, *t*-butylhydroperoxide, PhAsO, and others) that are known as potent promoters of PTP in most eukaryotic Ca^{2+}-transporting mitochondria [3, 5]. This was rather surprising as *E. magnusii* mitochondria, in contrast to mitochondria from other yeast species studied, have the high capacity, effectively regulated, uniporter driven Ca^{2+}-uptake system [19-26].

This could be due to: 1) lack of some component needed for the formation of a PTP; 2) different regulation of its opening, i.e., non-stimulation by Ca^{2+}, P_i, and prooxidants, or 3) presence of potent PTP opening inhibitors.

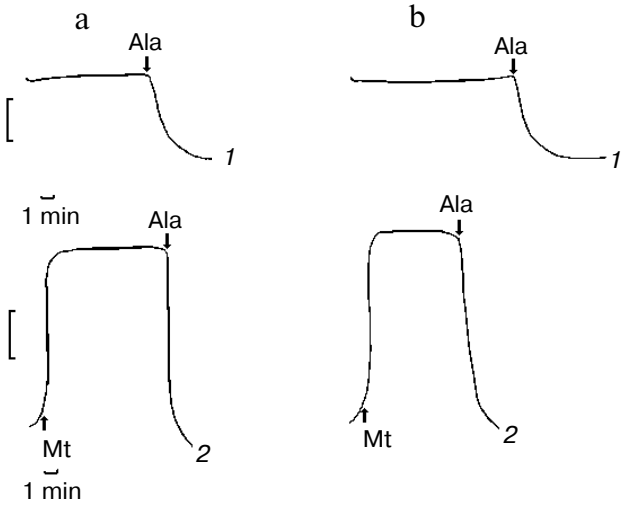

Fig. 7. Effect of alamethicin (Ala, 7.4 μg/ml) on swelling (*1*) and Δψ (*2*) of *E. magnusii* mitochondria in phosphate- (a) or acetate-containing medium (b). The experimental conditions for (a) were as in Fig. 1a, for (b) as in Fig. 4b. $A = 0.1$ (a) and 0.02 (b), respectively. Designations: Mt, mitochondria (0.5 mg protein).

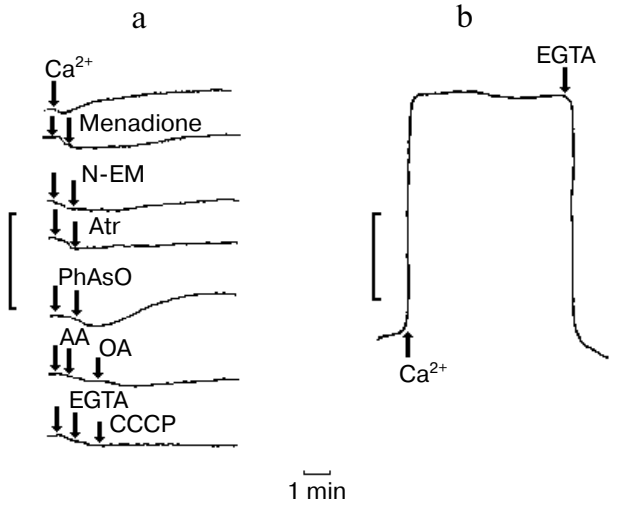

Fig. 8. Photometric recording of swelling (a) and uptake of 500 μM Ca^{2+} (b) by deenergized *E. magnusii* mitochondria. For composition of the incubation medium, see "Materials and Methods". Concentrations of PTP-triggering agents were as in Fig. 5. The Ca^{2+} uptake reaction (b) was terminated by addition of 0.5 M EGTA. $A = 0.1$ (a).

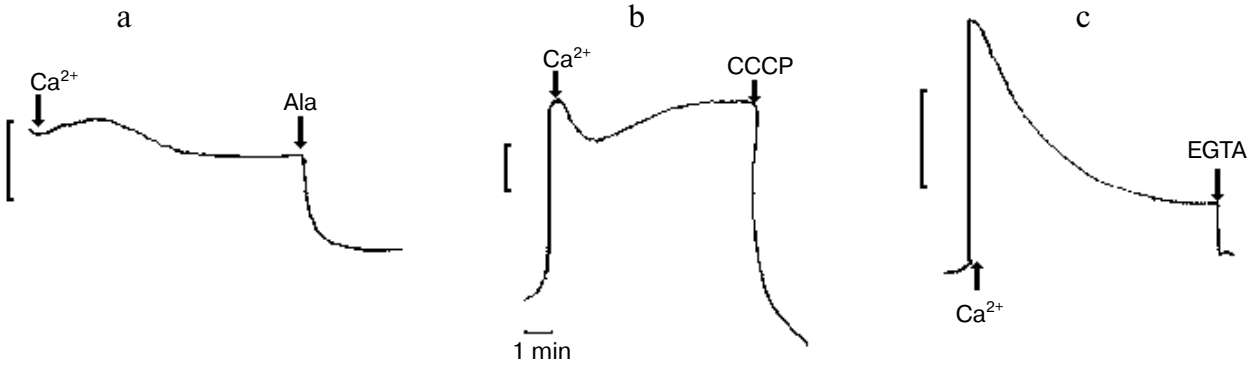

Fig. 9. Photometric recording of swelling (a), Δψ (b), and uptake of 500 μM Ca^{2+} (c) by *E. magnusii* mitochondria depleted of adenine nucleotides. The incubation medium was as in Fig. 4. Where indicated, alamethicin (Ala, 3.7 μg/ml), 1 μM CCCP, or 0.5 M EGTA was added. $A = 0.05$ (a) and 0.1 (b), respectively.

There are several components suggested to take part in PTP formation, though there is some uncertainty of which indeed are necessary [3]. One of these, cyclophilin D, a mitochondrial protein, the CsA receptor [48] that is engaged in the PTP development. A cyclophilin family with rather uncertain functions was found in yeast mitochondria. The only conclusively defined function is their involvement in the protein folding process [48]. It is conceivable that yeast mitochondrial cyclophilins may fulfill functions not related to the PTP, as all yeast mitochondria studied are not sensitive to CsA.

Yeast mitochondria [13, 19, 20], including those of *E. magnusii* [19, 20], are generally insensitive to high $[Ca^{2+}]$, which may be due to loss of the Ca^{2+}-sensor of the PTP [5]. It cannot be excluded that the matrix $[Ca^{2+}]$ in yeast mitochondria may be kept below the threshold by relatively high concentrations of P_i that precipitate the calcium [49]. There might also be a higher (compared to animal mitochondria) intramitochondrial concentrations of PTP inhibitors such as Mg^{2+} and/or spermine [7, 8]. Finally, the presence in yeast cells of the powerful antioxidant defense system [13, 16, 17], including thioredoxin peroxidase, two catalases, Cu^{2+}/Zn^{2+}- and Mn^{2+}-dependent superoxide dismutases (localized in the cytosol and mitochondria, respectively), cytochrome *c* peroxidase (localized in the intermembrane space), glu-

tathione and metallothionein [50], also may be responsible for the lack of the PTP in yeast mitochondria.

Deenergization of yeast mitochondria and depletion of their adenine nucleotides were found not to be responsible for membrane permeabilization (Figs. 8 and 9). Under these conditions, deenergized *E. magnusii* mitochondria did not undergo a membrane permeabilization in the presence of a number of potential PTP inducers, most probably because of almost total inhibition of the Ca^{2+} uptake via the energy-dependent uniporter (Fig. 8b). Mitochondria depleted of their adenine nucleotides were not permeabilized (Fig. 9), presumably because of lowering of matrix [Ca^{2+}] in the presence of high pyrophosphate concentrations.

Thus, *E. magnusii* mitochondria, possessing, in contrast to mitochondria of all other studied yeast species, separate, effectively regulated systems for Ca^{2+} influx and efflux [19-28], do not have the classical Ca^{2+}-dependent PTP. It means that the ability of mitochondria to take Ca^{2+} up by an energy-dependent manner and to control [Ca^{2+}] in the mitochondrial matrix as a result of concerted operation of pathways for Ca^{2+} uptake and efflux is not sufficient for PTP opening. Further studies are needed to ascertain why it is unfeasible to demonstrate opening of the classical PTP in yeast mitochondria. It is conceivable that yeast mitochondria are endowed by other mechanisms underlying regulation of ionic homeostasis such as ATP-dependent K^+-channel [18, 51, 52] and some others.

This study was supported by the Russian Academy of Sciences (grant on cellular and molecular biology) and by Russian Foundation for Basic Research (grants 03-04-48388 and 02-04-49717).

REFERENCES

1. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) *Nature*, **395**, 645-648.
2. Mooren, F. C., and Kinne, R. K. H. (1998) *Biochim. Biophys. Acta*, **1406**, 127-151.
3. Crompton, M. (1999) *Biochem. J.*, **341**, 233-249.
4. Pozzan, T., and Rizzuto, R. (2000) *Eur. J. Biochem.*, **267**, 5269-5273.
5. Bernardi, P. (1999) *Phys. Rev.*, **79**, 1127-1155.
6. Ichas, F., and Mazat, J.-P. (1998) *Biochim. Biophys. Acta*, **1366**, 33-50.
7. Novgorodov, S. A., Gudz, T. I., Brierley, G. P., and Pfeiffer, D. R. (1994) *Arch. Biochem. Biophys.*, **311**, 219-228.
8. Lapidus, R. G., and Sokolove, P. M. (1994) *J. Biol. Chem.*, **269**, 18931-18936.
9. Broekemeier, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989) *J. Biol. Chem.*, **264**, 7826-7830.
10. Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002) *Biochimie*, **84**, 153-166.
11. Samuilov, V. D., Oleskin, A. V., and Lagunova, E. M. (2000) *Biochemistry (Moscow)*, **65**, 873-887.
12. Proskuryakov, S. Y., Gabay, V. L., and Konoplyannikov, A. G. (2002) *Biochemistry (Moscow)*, **67**, 387-408.
13. Kowaltowski, A. J., Vercesi, A. E., Rhee, S. G., and Netto, L. E. S. (2000) *FEBS Lett.*, **473**, 177-182.
14. Carafoli, E., Balcavage, W. X., Lehninger, A. L., and Matton, J. R. (1970) *Biochim. Biophys. Acta*, **205**, 18-26.
15. Balcavage, W. X., Lloyd, J. L., Matton, J. R., Ohnishi, T., and Scarpa, A. (1973) *Biochim. Biophys. Acta*, **305**, 41-51.
16. Miranda-Vizuete, A., Damdimopoulos, A. E., and Spyrou, G. (2000) *Antioxid. Redox Signal.*, **2**, 801-810.
17. Garrido, E. O., and Grant, C. M. (2002) *Mol. Microbiol.*, **43**, 993-1003.
18. Jung, D. W., Bradshaw, P. C., and Pfeiffer, D. R. (1997) *J. Biol. Chem.*, **272**, 21104-21112.
19. Zvyagilskaya, R. A., Leykin, Y. N., Kozhokaru, N. L., and Kotelnikova, A. V. (1983) *Dokl. Akad. Nauk SSSR*, **269**, 1233-1240.
20. Leykin, Y. N., Votyakova, T. V., Bazhenova, E. N., Zvyagilskaya, R. A., and Kotelnikova, A. V. (1987) *Biokhimiya*, **52**, 676-682.
21. Votyakova, T. V., Bazhenova, E. N., and Zvjagilskaya, R. A. (1990) *FEBS Lett.*, **261**, 139-141.
22. Votyakova, T. V., Bazhenova, E. N., and Zvjagilskaya, R. A. (1993) *J. Bioenerg. Biomembr.*, **25**, 569-574.
23. Deryabina, Y. I., Bazhenova, E. N., and Zvyagilskaya, R. A. (1996) *Biochemistry (Moscow)*, **61**, 1206-1212.
24. Bazhenova, E. N., Deryabina, Y. I., and Zvyagilskaya, R. A. (1997) *Dokl. Akad. Nauk SSSR*, **353**, 1-3.
25. Bazhenova, E. N., Saris, N.-E. L., and Zvyagilskaya, R. A. (1998) *Biochim. Biophys. Acta*, **1371**, 96-100.
26. Bazhenova, E. N., Deryabina, Yu. I., Eriksson, O., Zvyagilskaya, R. A., and Saris, N.-E. L. (1998) *J. Biol. Chem.*, **273**, 4372-4377.
27. Deryabina, Y. I., and Zvyagilskaya, R. A. (2000) *Biochemistry (Moscow)*, **65**, 1352-1356.
28. Deryabina, Y. I., Bazhenova, E. N., Saris, N. E., and Zvyagilskaya, R. A. (2001) *J. Biol. Chem.*, **276**, 47801-47806.
29. Zvyagilskaya, R. A., and Sholtz, C. F. (1980) in *Biochemical Methods* (Kretovich, V. L., and Sholtz, K. F., eds.) [in Russian], Nauka, Moscow, pp. 13-18.
30. Zvyagilskaya, R. A., Zelentshchikova, V. A., and Kotelnikova, A. V. (1981) *Biokhimiya*, **46**, 3-10.
31. Asimakis, G. K., and Sordahl, L. A. (1981) *Am. J. Physiol.*, **241**, H672-H678.
32. Connern, C. P., and Halestrap, A. P. (1994) *Biochem. J.*, **302**, 321-324.
33. Scarpa, A. (1979) *Meth. Enzymol.*, **56**, 301-338.
34. Akerman, K. E. O., and Wikstrom, M. K. F. (1976) *FEBS Lett.*, **68**, 191-197.
35. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
36. Halestrap, A. P., and Davidson, A. M. (1990) *Biochem. J.*, **268**, 153-160.
37. Chavez, E., and Osornio, A. (1988) *Int. J. Biochem.*, **20**, 731-736.
38. Petronilli, V., Nicolli, A., Costantini, P., Colonna, P., and Bernardi, P. (1994) *Biochim. Biophys. Acta*, **1187**, 255-259.
39. Gerasimenko, J. V., Gerasimenko, O. V., Palejwala, A., Tepikin, A. V., Petersen, O. H., and Watson, A. J. (2002) *J. Cell Sci.*, **115**, 485-497.
40. He, L., and Lemasters, J. (2002) *FEBS Lett.*, **512**, 1-7.

41. Bernardi, P., Veronese, P., and Petronilli, V. (1993) *J. Biol. Chem.*, **268**, 1005-1010.
42. Petronilli, V., Cola, C., and Bernardi, P. (1993) *J. Biol. Chem.*, **268**, 1011-1016.
43. Palmer, J. W., and Pfeiffer, D. R. (1981) *J. Biol. Chem.*, **256**, 6742-6750.
44. Igbavboa, U., and Pfeiffer, D. R. (1988) *J. Biol. Chem.*, **263**, 1405-1412.
45. Lapidus, R. G., and Sokolove, P. M. (1993) *Arch. Biochem. Biophys.*, **306**, 246-253.
46. Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M., and Bernardi, P. (1996) *J. Biol. Chem.*, **271**, 2185-2192.
47. Harris, P. I., Molle, G., and Duclohier, H. (2004) *Biophys. J.*, **86**, 248-253.
48. Matoushek, A., Rospert, S., Schmid, K., Glick, B. S., and Schatz, G. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 6319-6323.
49. Chavez, E., Moreno-Sanchez, R., Zazueta, C., Rodríguez, J. S., Bravo, C., and Reyes-Vivas, C. H. (1997) *J. Bioenerg. Biomembr.*, **29**, 571-577.
50. Jamieson, D. J. (1998) *Yeast*, **14**, 1511-1527.
51. Roucou, X., Manon, S., and Guerin, M. (1995) *FEBS Lett.*, **364**, 161-164.
52. Manon, S., and Guerin, M. (1997) *Biochim. Biophys. Acta*, **1318**, 317-321.