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Channel Formation by Yeast F-ATP Synthase and the Role of Dimerization in the Mitochondrial Permeability Transition*†‡

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Background: Whether channel formation is a general feature of F-ATP synthase dimers across species is unknown.

Results: Yeast F-ATP synthase dimers form Ca2+-dependent channels, and the e and g subunits facilitate pore formation in situ through dimerization.

Conclusion: F-ATP synthase dimers form the permeability transition pore of yeast.

Significance: Ca2+-dependent channel formation is a conserved feature of F-ATP synthases.

Purified F-ATP synthase dimers of yeast mitochondria display Ca2+-dependent channel activity with properties resembling those of the permeability transition pore (PTP) of mammals. After treatment with the Ca2+ ionophore ETH129, which allows electrophoretic Ca2+ uptake, isolated yeast mitochondria undergo inner membrane permeabilization due to PTP opening. Yeast mutant strains ΔTIM11 and ΔATP20 (lacking the e and g F-ATP synthase subunits, respectively, which are necessary for dimer formation) display a striking resistance to PTP opening. These results show that the yeast PTP originates from F-ATP synthase dimers of yeast mitochondria. 

Mitochondria from a variety of sources can undergo an inner membrane permeability increase, the permeability transition (PT), due to opening of a high conductance channel, the PT pore (PTP) (1). The PTP coincides with the mitochondrial megachannel (MMC) defined by patch clamp studies in mitochondria from yeast, where multiple conductance pathways may exist including an uncoupling protein–independent permeability activated by ATP (15–17). Furthermore, the yeast PTP (yPTP) is inhibited rather than activated by Ps and insensitive to CsA (9), and due to the lack of a mitochondrial Ca2+ uniporter, its Ca2+ dependence has been more difficult to assess (18), although the Ca2+ content of Saccharomyces cerevisiae mitochondria is close to that of rat liver mitochondria (19). The problem of the Ca2+ dependence was solved by the Shinohara group (20), who showed that yeast mitochondria incubated with optimized substrates and P, concentrations readily undergo a Ca2+-dependent PT upon treatment with ETH129, a Ca2+ ionophore that allows electrophoretic Ca2+ transport into the matrix of energized mitochondria. We recently demonstrated that dimers of mammalian F-ATP synthase reconstituted into planar bilayers give rise to Ca2+-activated currents with conductances ranging up to 1.3 nS in 150 mM KCl that closely match those displayed by the MMC-PTP (21). Here we have tested whether gel-purified F-ATP synthase dimers of S. cerevisiae form channels when reconstituted in lipid bilayers, and whether dimerization of the F-ATP synthase is necessary for PTP formation in intact mitochondria.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Materials—**The S. cerevisiae strains BY4743 (4741/4742), as well as the mutants ΔCPR3 (MATa, his3Δ1, leu2Δ0, met5Δ0, ura3Δ0), ΔTIM11 (MATα, his3Δ1, leu2Δ0, met5Δ0, ura3Δ0), and ΔATP20 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0), were purchased from Thermo Scientific. ΔTIM11ΔATP20 mutants were obtained by mating the ΔTIM11 and ΔATP20 strains and selecting the formed diploid by growth on synthetic defined (0.67% nitrogen base without amino acids, 2% dextrose) selective medium containing the

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required nutritional supplements except methionine and lysine. Diploids were then induced to sporulate in 1% potassium acetate, tetrads were dissected, and haploids were analyzed with semiquantitative PCR to detect null mutants for TIM11 and ATP20 genes. Digitonin was from Sigma, and ETH129 was from Sigma-Aldrich Japan and was dissolved in methanol. NADH, disodium salt was purchased from Roche Applied Science.

Yeast Culture and Mitochondria Isolation—Yeast cells were cultured aerobically in 50 ml of 1% yeast extract, 1% bacto-polypeptone medium containing 2% glucose at 30 °C. When it reached an optical density of 2 at 600 nm, the culture was added to 800 ml of bacto-polypeptone medium supplemented with 2% galactose and incubated for 20 h at 30 °C under rotation at 180 rpm, yielding about 4.0 g of yeast cells. Yeast mitochondria were isolated as described (20) with the following modifications. Briefly, cells were washed, incubated for 15 min at 37 °C in a 0.1 M Tris-SO4 buffer (pH 9.4) supplemented with 10 mM dithiothreitol (DTT), and washed once with 1.2 M sorbitol, 20 mM P, pH 7.4. Yeast cells were then suspended in the same buffer and incubated for 45 min at 30 °C with 0.4 mg/ml of cells of Zymolyase 100T to form spheroplasts. The latter were washed once with sorbitol buffer and homogenized in 0.6 M Mannitol, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA-Tris with a Potter homogenizer. The homogenate was centrifuged for 5 min at 2,000 × g, and the supernatant was collected and centrifuged for 10 min at 12,000 × g. The resulting mitochondrial pellet was suspended in mannitol buffer, and protein concentration was determined from the A280 of SDS-solubilized mitochondria (14).

Mitochondrial Calcium Retention Capacity—Mitochondrial Ca2+ uptake was measured with Calcium Green-5N (Molecular Probes) fluorescence using a Fluoroskan Ascent FL (Thermo Electron) plate reader at a mitochondrial concentration of 0.5 mg × ml−1. Mitochondria were incubated as specified in the figure legends.

**Results and Discussion**

**Properties of the Ca2+-dependent Permeability Transition of Yeast Mitochondria**—We used ETH129 to allow Ca2+ uptake by energized yeast mitochondria (20) and monitored the propensity of the yPTP to open based on the Ca2+ retention capacity (CRC), i.e. the maximal Ca2+ load retained by mitochondria before onset of the PT (22). In keeping with previous observations (20), (i) energized yeast mitochondria were able to accumulate Ca2+ provided as a train of pulses (Fig. 1A) until onset of the PT, which causes depolarization followed by Ca2+ release; and (ii) increasing concentrations of P, increased the matrix Ca2+ load necessary to open the yPTP (Fig. 1, A and B), possibly following formation of matrix P,Ca2+ complexes. As in mammalian mitochondria, Mg2+-ADP increased the CRC, an effect consistent with yPTP inhibition (Fig. 1C). The CRC was not affected by decavanadate (results not shown), which inhibits the ATP-induced, voltage-dependent anion channel (VDAC)-dependent yeast permeability pathway (23, 24).

The mammalian PTP is modulated by two classes of redox-sensitive thiols whose oxidation increases the pore sensitivity to Ca2+, i.e. (i) matrix thiols that react with phenylarsine oxide (PhAsO) and can be oxidized by diamide (25); and (ii) external thiols that can be oxidized by copper-o-phenanthroline (Cu(OOP)2) (26). The threshold Ca2+ load required for yPTP opening was moderately affected by PhAsO (Fig. 1D), whereas it was very sensitive to diamide (Fig. 1E) and to Cu(OOP)2 (Fig. 1F). These experiments indicate that the yeast PTP is affected by the redox state of thiol groups as also suggested by a previous study (18).

CsA desensitizes the mammalian pore to Ca2+ through matrix CyPD, a peptidyl-prolyl cis-trans isomerase that behaves as a PTP inducer (27, 28). Through studies of CyPD-null mitochondria, it became clear that CyPD is a modulator but not an obligatory constituent of the PTP and that a PT can occur in the

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absence of CyPD, or in the presence of CsA, albeit at higher matrix Ca^{2+} loads (8). Yeast mitochondria possess a matrix CyP (CPR3), which facilitates folding of imported proteins in the matrix and is sensitive to CsA (29); however, the yPTP is not affected by CsA (9), as also confirmed in the CRC assay (Fig. 2A, compare traces a and b). These findings suggest either that CPR3 does not interact with the pore or that CsA does not interfere with CPR3 binding. To resolve this issue, we tested the CRC of ΔCPR3 mutants, which displayed a lower rate and slightly lower extent of Ca^{2+} accumulation (Fig. 2A, trace c), indicating that CPR3 does not sensitize the yPTP to Ca^{2+}, at variance from the effects of CyPD in mammalian mitochondria (30). The small decrease of CRC in the mutants (Fig. 2B) may be due to slower protein import and defective respiratory chain assembly and/or function (31). It was recently established that rotenone is a good inhibitor of the PTP in mammalian mitochondrial protein extracts by BN-PAGE, identified dimers in symmetrical 150 mM KCl did not elicit current activity unless Ca^{2+}, PhAsO, and Cu(OP)_{2} were also added (Fig. 3A). We observed a clear activity in 12 out of 14 reconstitutions, with
channel unit conductance usually ranging between 250 and 300 pS (multiples of this unit conductance were often observed; in one case 1000 pS was reached). This conductance is compatible with the values exhibited by a channel observed in mitoplasts from a porin-less yeast strain, which was insensitive to CsA, ADP, or protons and in which the combination of ADP and Mg$_{2+}$ was not tested (33). The activity studied here was characterized by rapid oscillations between closed and open states (fickering), which is typical of the mammalian MMC-PTP, and by variable kinetics. A typical fickering behavior is illustrated in the bottom part of Fig. 3A. As is the case for the mammalian F-ATP synthase (21) and for the MMC-PTP measured in mitoplasts (4), the addition of Mg$_{2+}$-ADP induced a clear-cut inhibition of the channel in five out of six experiments (total inhibition was observed in two cases, and partial inhibition was observed in three cases). The representative experiment of Fig. 3B shows activity recorded before and immediately after the addition of Mg$_{2+}$-ADP in one case of full inhibition, which is illustrated in the corresponding amplitude histograms (Fig. 3B). Taken together, these data provide evidence that under conditions of oxidative stress, yeast F-ATP synthase can form Ca$_{2+}$-activated channels with features resembling the MMC-PTP (although with lower conductance). It should be noted that the dimer preparation did not contain Tom20 or Tim54 (Fig. 4A) and therefore that channel activity cannot be due to the twin pore translocase (34).

**Dimerization of F-ATP Synthase Is Required for PTP Formation**—Dimers of F-ATP synthase are the “building blocks” of long rows of oligomers located deep into the cristae, which contribute to formation of membrane curvature and to maintenance of proper cristae shape and mitochondrial morphology (35–42). Mammalian F-ATP synthase dimers also appear to be the units from which the PTP forms in a process that is highly favored by Ca$_{2+}$+ and oxidative stress (21), events that are required for channel formation (8, 21). To test the hypothesis that yPTP formation requires the presence of F-ATP synthase dimers, we studied mutants lacking subunits involved in dimerization/oligomerization of the enzyme, i.e. subunit e (TIM11) and subunit g (ATP20) (35, 43–45). Strains lacking these subunits display balloon-shaped cristae with ATP synthase monomers distributed randomly in the membrane (39). The ΔTIM11, ΔATP20, and ΔTIM11ΔATP20 mutants lacked dimers when analyzed by BN-PAGE, whereas the monomeric F-ATP synthase was assembled and active (Fig. 4A), consistent with their ability to grow on non-fermentable carbon sources, and developed a normal membrane potential upon energization with NADH (results not shown). CRC assays with ETH129 demonstrated that mitochondria from ΔTIM11, ΔATP20, and ΔTIM11ΔATP20 strains take up a larger Ca$_{2+}$ load than wild-type strains (Fig. 4B), with a doubling of the CRC (Fig. 4C).

Dimers may transiently form also in ΔTIM11 and ΔATP20 strains (46), a finding that could explain why Ca$_{2+}$+ release is eventually observed also in the “dimerization-less” mutants. Consistent with this possibility, we did detect dimers in BN-PAGE after treatment with CuCl$_2$ (Fig. 4D), which promotes formation of disulfide bridges between adjacent cysteine residues of the monomers (45, 47, 48). Not all of the monomers dimerized after CuCl$_2$ treatment (Fig. 4D), suggesting that cysteine oxidation stabilizes pre-existing dimers that are otherwise
dissociated by detergent treatment, but does not induce cross-linking of monomers.

In summary, our data provide the first demonstration that yeast F-ATP synthase dimers form high conductance channels analogous to the mammalian MMC-PTP, and thus that channel formation is a conserved feature of F-ATP synthases; show that yeast mitochondria can undergo a bona fide PT activated by oxidative stress; and indicate that dimers of F-ATP synthase conferring protein.

REFERENCES


