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

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Running title: Channel formation by yeast F-ATP synthase

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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 Ca\textsuperscript{2+}-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: Ca\textsuperscript{2+}-dependent channel formation is a conserved feature of F-ATP synthases.

Purified F-ATP synthase dimers of yeast mitochondria display Ca\textsuperscript{2+}-dependent channel activity with properties resembling those of the permeability transition pore (PTP) of mammals. After treatment with the Ca\textsuperscript{2+} ionophore ETH129, which allows electrophoretic Ca\textsuperscript{2+} 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, and indicate that dimerization is required for pore formation in situ.

Mitochondria from a variety of sources can undergo an inner membrane permeability increase, the permeability transition (PT\textsuperscript{3}), 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 mitoplasts (2-5). In mammals PTP opening requires matrix Ca\textsuperscript{2+}, is favored by oxidative stress and Pi, inhibited by adenine nucleotides and Mg\textsuperscript{2+}, and antagonized by cyclosporin A (CsA) through its interaction with matrix cyclophylin (CyP)D (6,7). The mammalian PTP is today recognized to play a role in cell death in a variety of disease paradigms (8).

Inner membrane permeability pathways have been described in yeast (9) and in Drosophila melanogaster (10) but whether these coincide with the mammalian PTP remains an open question (11-14). The issue is particularly complex in the case of yeast, where multiple conductance pathways may exist including an UCP-independent permeability activated by ATP (15-17). Furthermore, the yeast PTP (yPTP) is inhibited rather than activated by Pi, insensitive to CsA (9) and, due to the lack of a mitochondrial Ca\textsuperscript{2+} uniporter, its Ca\textsuperscript{2+}-dependence has been more difficult to assess (18) although the Ca\textsuperscript{2+} content of Saccharomyces cerevisiae mitochondria is close to that of rat liver mitochondria (19). The problem of the Ca\textsuperscript{2+} dependence was solved by Shinohara’s group, who showed that yeast mitochondria incubated with optimized substrate and Pi concentrations readily undergo a Ca\textsuperscript{2+}-dependent PT upon treatment with ETH129, a Ca\textsuperscript{2+} ionophore that allows electrophoretic Ca\textsuperscript{2+} transport into the matrix of energized mitochondria (20). We recently demonstrated that dimers of mammalian F-ATP synthase reconstituted into planar bilayers give rise to Ca\textsuperscript{2+}-dependent channel formation.
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 *Saccharomyces cerevisiae* forms 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 (MATa, his3Δ1, leu2Δ0, met5Δ0, ura3Δ0) and ΔATP20 (MATa, 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 SD (0.67% nitrogen base w/o amino acids, 2% dextrose) selective medium containing the 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 semi-quantitative PCR to detect null mutants for TIM11 and ATP20 genes. Digitonin was from Sigma, and ETH129 from Sigma Aldrich Japan, and was dissolved in methanol. NADH, disodium salt was purchased from Roche.

**Yeast culture and mitochondria isolation** - Yeast cells were cultured aerobically in 50 ml of 1% yeast extract, 1% bacto-polypeptone (YP) 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 YP medium supplemented with 2% galactose and incubated for 20 h at 30°C to induce sporulation. 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 Pi pH 7.4. Yeast cells were then suspended in the same buffer and incubated for 45 min at 30°C with 0.4 mg/g of cells of zymolase 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 x g, the supernatant was collected and centrifuged for 10 min at 12,000 x g. The resulting mitochondrial pellet was suspended in mannitol buffer and protein concentration was determined from the A_{280} 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 x ml⁻¹. Mitochondria were incubated as specified in the figure legends.

**Gel electrophoresis and Western blotting** - Mitochondria were suspended at 10 mg/ml in 150 mM K-acetate, 30 mM HEPES, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and solubilized with 1.5% (w/v) digitonin. After centrifugation at 100,000 x g with a Beckman TL-100 rotor for 25 min at 4°C, supernatants were collected, supplemented with 50 mg/ml Coomassie Blue and 5 M aminoacaproic acid and quickly loaded onto a blue native polyacrilamide 3-12% gradient gel (BN-PAGE, Invitrogen). Electrophoresis was carried out at 150V for 20 min and at 250V for 2 h, followed by gel staining with 0.25 mg/ml Coomassie Blue, 10% acetic acid or used for in-gel activity staining to detect bands corresponding to ATP synthase. Activity was monitored in 270 mM glycine, 35 mM Tris pH 7.4, 15 mM MgSO₄, 8 mM ATP-Tris, and 2 mg/ml Pb(NO₃)₂. Bands corresponding to monomeric and dimeric forms of ATP synthase were cut from the gels and protein complexes were eluted overnight by incubation at 4°C in 25 mM Tricine, 15 mM MgSO₄, 8 mM ATP, 7.5 mM Bis-Tris, 1% (w/v) n-heptyl β-D-thioglucopyranoside, pH 7.0. Samples were then centrifuged at 20,000 x g for 10 min at 4°C and supernatants were used for bilayer experiments. For cross-linking experiments, mitochondria were incubated 20 min at room temperature at 1 mg/ml in 250 mM sucrose, 2 mM Pi and 2 mM CuCl₂. Five millimolar N-ethylmaleimide and 5 mM EDTA were then added to block the cross-linking reaction, the incubations transferred on ice for 10 min followed by centrifugation and preparation for BN-PAGE as described above. Total yeast mitochondria lysates and bands corresponding to dimers of F-ATP synthase cut out of BN-PAGE gels were subjected to SDS-PAGE followed by silver staining or transfer to nitrocellulose for Western blot analysis. Antibodies were polyclonal rabbit anti-ATP synthase γ subunit (a gift from...
Marie-France Giraud, Bordeaux), anti-Tom20 and anti-Tim54 (a gift from Nikolaus Pfanner, Freiburg).

Electrophysiology - Planar lipid bilayer experiments were performed as described in (31). Briefly, bilayers of 150-200 pF capacitance were prepared using purified soybean asolectin. The standard experimental medium was 150 mM KCl, 10 mM Hepes, pH 7.5. All reported voltages refer to the cis chamber, zero being assigned to the trans (grounded) side. Currents are considered as positive when carried by cations flowing from the cis to the trans compartment. Freshly prepared F-ATP synthase dimers were added to the cis side. No current was observed when PTP activators were added to the membrane in the absence of F-ATP synthase dimers (n = 2).

RESULTS AND DISCUSSION

Properties of the Ca$^{2+}$-dependent permeability transition of yeast mitochondria - We used ETH129 to allow Ca$^{2+}$ uptake by energized yeast mitochondria (20), and monitored the propensity of the yPTP to open based on the Ca$^{2+}$ retention capacity (CRC), i.e. the maximal Ca$^{2+}$ load retained by mitochondria before onset of the PT (22). In keeping with previous observations (20) (i) energized yeast mitochondria were able to accumulate Ca$^{2+}$ provided as a train of pulses (Fig 1A) until onset of the PT, which causes depolarization followed by Ca$^{2+}$ release; and (ii) increasing concentrations of Pi increased the matrix Ca$^{2+}$ load necessary to open the yPTP (Fig 1A,B), possibly following formation of matrix Pi-Ca$^{2+}$ complexes. Like in mammalian mitochondria Mg$^{2+}$-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, VDAC-dependent yeast permeability pathway (23,24).

The mammalian PTP is modulated by 2 classes of redox-sensitive thiols whose oxidation increases the pore sensitivity to Ca$^{2+}$, i.e. (i) matrix thiols that react with phenylarsine oxide (PhAsO) and can be oxidized by diame (25); and (ii) external thiols that can be oxidized by copper-o-phenanthroline (Cu(OP)$_2$) (26). The threshold Ca$^{2+}$ load required for yPTP opening was moderately affected by PhAsO (Fig 1D) while it was very sensitive to diame (Fig 1E) and to Cu(OP)$_2$ (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 Ca$^{2+}$ through matrix CyP, 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 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); yet 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 that either 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 ACPR3 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 mitochondria lacking CyPD, possibly because of decreased production of reactive oxygen species through inhibition of reverse electron flow (32). Rotenone did not affect the yPTP (Fig 2A, trace d), in keeping with the lack of a rotenone-sensitive, energy-conserving complex I and with the lack of "off-site" effects. Taken together, the above results suggest that, in spite of the lack of a fast Ca$^{2+}$ uptake system (19), S. cerevisiae mitochondria can undergo a Ca$^{2+}$-induced PT which displays some similarities with the mammalian PT (sensitization by matrix Ca$^{2+}$ and oxidative stress, inhibition by Mg$^{2+}$-ADP), but also some differences (inhibition by phosphate, lack of sensitivity to CPR3 and rotenone).

Purified F-ATP synthase dimers possess channel activity - To test whether yeast F-ATP synthase dimers can form channels similar to those found in mammals (21), we separated mitochondrial protein extracts by BN-PAGE, identified dimers by in-gel activity staining, and eluted them for incorporation into a planar asolectin membrane (an example of the dimer used can be found in Fig 4A). Addition of 1-10 pmol of the dimers to the bilayers 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 (flickering), which is typical of the mammalian MMC-PTP, and by variable kinetics. A typical flickering 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), addition of Mg\(^{2+}\)-ADP induced a clear-cut inhibition of the channel in 5 out of 6 experiments (total inhibition was observed in 2 cases and partial inhibition in 3 cases); the representative experiment of Fig 3B shows activity recorded before and immediately after 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 appear also 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 \(\Delta TIM11\), \(\Delta ATP20\) and \(\Delta TIM11\Delta ATP20\) mutants lacked dimers when analyzed by BN-PAGE while 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 \(\Delta TIM11\), \(\Delta ATP20\) and \(\Delta TIM11\Delta ATP20\) 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 \(\Delta TIM11\) and \(\Delta ATP20\) 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 are required for PTP formation in situ (21). Our findings do not exclude the existence of other permeability pathways which may involve VDAC (23,24), nor the possible regulation of yPTP by outer mitochondrial membrane proteins (8). We think that it will now be possible to unravel the many open questions about the structure and function of the PTP (8) with the powerful methods of yeast genetics.
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The abbreviations used are: BN-PAGE, blue native polyacrylamide gel electrophoresis; CRC, Ca2+ retention capacity; Cu(OP)2, copper-o-phenanthroline; CsA, cyclosporin A; CyPD, cyclophilin D; DTT, dithiothreitol; MMC, mitochondrial megachannel; PhAsO, phenylarsine oxide; PT, permeability transition; PTP, permeability transition pore; yPTP, yeast permeability transition pore.
FIGURE LEGENDS

FIGURE 1. Properties of the permeability transition of yeast mitochondria. The incubation medium contained 250 mM Sucrose, 10 mM Tris-MOPS, 1 mM NADH, 10 μM EGTA- Tris, 5 μM ETH129, 1 μM Calcium Green-5N, final pH 7.4, 0.5 mg/ml bovine serum albumin and 0.1 mg of mitochondria in a final volume of 0.2 ml. (A) The medium was supplemented with 1 mM (trace a), 2 mM (trace b), 5 mM (trace c) or 10 mM Pi (trace d), and where indicated Ca^{2+} was added; traces shown are representative of 13 independent experiments. (B) Experimental conditions as in panel A with the indicated Pi concentrations; values on the ordinate refer to the amount of Ca^{2+} accumulated prior to the precipitous release that follows the PT (n = 13 ± SE). (C) The experimental conditions were as in panel A with 2 mM Pi, and the medium supplemented with 2 mM MgCl_{2}, 1 μM oligomycin and the stated concentrations of ADP (n = 8 ± SE). (D-F) The experimental conditions were as in panel A with 2 mM Pi, and the medium supplemented with the stated concentrations of PhAsO (D), diamide (E) or Cu(OP)_{2} (F). For panels D-F n (± SE) was 6, 4 and 7, respectively.

FIGURE 2. CPR3 deletion does not affect the yeast permeability transition. (A) The experimental conditions were as in Fig 1 with 2 mM Pi; 0.8 μM CsA was added in trace b only and 2 μM rotenone in trace d only; where indicated Ca^{2+} was added to wild-type (traces a,b,d) or ΔCPR3 (trace c) mitochondria (traces are representative of 3 independent experiments). (B) The experimental conditions were as in Fig 1 with Pi as indicated (n = 4 ± SE). Closed symbols, wild-type mitochondria; open symbols, ΔCPR3 mitochondria. Two-Way ANOVA test was performed, *P<0.05.

FIGURE 3. F-ATP synthase dimers reconstituted in planar lipid bilayers display Ca^{2+}-induced currents. Dimers were excised (see Fig 4A, wild-type) and eluted for planar bilayer experiments. (A) Upper part: Representative current traces recorded at +80 and -100 mV (cis) (upper and lower traces, conductance (g) = 125 and 250 pS) upon incorporation of purified dimeric F-ATP synthase following addition of 3 mM Ca^{2+} (added to the trans side) plus 0.1 mM PhAsO and 20 μM Cu(OP)_{2} (added to both sides). Lower part: typical, most often observed channel kinetics (see also expanded portion of the recording obtained at -60 mV (cis); g = 250 pS). (B) Top: Effect of 2 mM ADP plus 1.6 mM Mg^{2+} added to the trans side on channel activity (-60 mV, g = 250 pS); current trace before and immediately after addition of the modulators is shown; bottom, amplitude histograms obtained from the same experiment before (left panel) and after (right panel) addition of ADP/Mg^{2+}. Gaussian fitting (green lines) was obtained using the Origin 6.1 Program Set.

FIGURE 4. ΔTIM11, ΔATP20 and ΔTIM11ΔATP20 mutants lacking subunits involved in dimerization of F-ATP synthase are resistant to PTP opening. (A) Mitochondrial protein extracts were separated with BN-PAGE and stained with Coomassie blue (lanes labeled Coomassie) or subjected to in-gel activity staining (lanes labeled Activity) to identify bands of F-ATP synthase dimers and monomers (note also a faint band corresponding to F_{i}). The gel region corresponding to the dimers of the BY4743 strain was cut out and subjected to SDS-PAGE together with a mitochondrial extract from the same strain, followed by silver staining (lanes labeled Silver staining) or blotting and probing with the indicated antibodies (lanes labeled Western). (B) The experimental conditions were as in Fig 1 with 1 mM Pi; where indicated Ca^{2+} was added to wild-type, ΔTIM11ΔATP20, ΔTIM11 or ΔATP20 mutants (traces are representative of 13, 6, 7 and 6 independent experiments for the corresponding genotypes). (C) Experimental conditions as in (A) with 1 mM Pi. One-Way ANOVA test was performed to analyze CRC differences between BY4743 and mutants, *P<0.01, **P<0.001. (D) BN-PAGE (left lanes) and activity staining (right lanes) of mitochondria with the indicated genotypes after treatment with 2 mM CuCl_{2}. 

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Figure 1
Figure 2

(A) Ca²⁺ Green-5N fluorescence, a.u.

- a: 20 μM Ca²⁺ each

(B) CRC (nmol x mg⁻¹)

- x axis: Pi, mM
- y axis: CRC

5 min

*
Figure 3
Figure 4

Coomassie Activity Silver staining

** CRC (nmol x mg⁻¹) C
B Ca²⁺ Green-5N fluorescence, a.u.

20 μM Ca²⁺ each

Figure 4
Channel Formation by Yeast F-ATP Synthase and the Role of Dimerization in the Mitochondrial Permeability Transition
Michela Carraro, Valentina Giorgio, Justina Sileikyte, Geppo Sartori, Michael Forte, Giovanna Lippe, Mario Zoratti, Ildikò Szabò and Paolo Bernardi

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