The Key Role of the Energized State of *Saccharomyces cerevisiae* Mitochondria in Modulations of the Outer Membrane Channels by the Intermembrane Space Proteins

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Mitochondria of the yeast *Saccharomyces cerevisiae* constitute a perfect model to study the outer membrane channel modulation as besides the TOM complex channel they contain only a single isoform of the VDAC channel and it is possible to obtain viable mutants devoid of the channel. Here, we report that the fraction of the intermembrane space isolated from wild type and the VDAC channel-depleted yeast mitochondria, except of the well-known VDAC channel modulator activity, displays also the TOM complex channel modulating activity as measured in the reconstituted system and with intact mitochondria. The important factor influencing the action of both modulating activities is the energized state of mitochondria. Moreover, the presence of the VDAC channel itself seems to be crucial to properties of the intermembrane space protein (s) able to modulate the outer membrane channels because in the case of intact mitochondria quantitative differences are observed between modulating capabilities of the fractions isolated from wild type and mutant mitochondria.

KEY WORDS: Mitochondria; the TOM complex channel; the VDAC channel; the VDAC channel modulator fraction; modulation.

INTRODUCTION

It is well known that transport of molecules across the outer mitochondrial membrane is essential for mitochondrial physiology. The transported molecules are not only metabolites exchanged between mitochondria and cytosol but also proteins imported into mitochondria. In spite of the functional complexity, channels being the permeability pathways of the outer membrane seem to be quite limited in their diversity. The TOM complex channel (channel of the translocase of the outer membrane) enables protein translocation (Ahting *et al.*, 1999, 2001; Hill *et al.*, 1998; Künkele *et al.*, 1998a,b; Meisinger *et al.*, 1999) while the flux of metabolites is supported by the VDAC channel (voltage-dependent anion-selective channel), also known

as mitochondrial porin (Benz, 1994; Blachly-Dyson and Forte, 2001; Colombini *et al.*, 1996). The latter, however, may be present as isoforms encoded by separated genes and displaying different channel-forming activities (Blachly-Dyson *et al.*, 1993, 1997; Elkeles *et al.*, 1997; Heins *et al.*, 1994; Sampson *et al.*, 1997; Xu *et al.*, 1999).

The existence of mitochondrial proteins being able to modulate functional states of the outer membrane channels is probably crucial to the membrane permeability. In the case of the TOM complex channel, proteins able to modulate the gating mechanisms were reported to be components of the complex (Meisinger *et al.*, 2001; Rapaport *et al.*, 1998; Van Wilpe *et al.*, 1999) or translocated polypeptide chains (Künkele *et al.*, 1998b; Rapaport *et al.*, 1998). Known regulators of the VDAC channel are pro- and antiapoptotic BCL-2 family members Gross, McDonnell and Korsmeyer (1999). However, data accessible at present do not allow to clearly explain the role of the proteins in VDAC channel modulation (Crompton *et al.*, 2002; Shimizu *et al.*, 2000; Tsujimoto and Shimizu, 2002; Vander Heiden *et al.*, 1999, 2000). On the other hand

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the VDAC channel modulating activities, often referred to as the VDAC channel modulator, were proved to exist in the intermembrane space of mitochondria although responsible protein (s) has not been identified (Elkeles *et al.*, 1997; Holden and Colombini, 1988, 1993; Liu *et al.*, 1994; Liu and Colombini, 1992). The VDAC channel modulator was studied mainly in reconstituted systems (Elkeles *et al.*, 1997; Holden and Colombini, 1988, 1993; Liu *et al.*, 1994) but also with intact mitochondria (Liu and Colombini, 1992). In reconstituted systems, it increases the voltage dependence of VDAC channels in three ways: it increases the rate of channel closure, decreases the rate of opening, and induces the channels to assume lower conductance closed states. With intact mitochondria, the VDAC channel modulator was shown to partially inhibit transport of adenine nucleotides across the outer membrane when added to the cytoplasmic side. Thus, the VDAC channel modulator seems to mediate changes of VDAC channel conductance states also in native mitochondria. Since it has been shown recently that the TOM complex channel is able to support metabolite transport in the absence of the VDAC channel as well as under conditions of its insufficient permeability (Antos *et al.*, 2001a,b; Kmita and Budzińska, 2000), the question arises whether the VDAC channel modulator may also participate in the regulation of the TOM complex channel. The second problem that is interesting to study is the relation between the presence of the VDAC channel and expression of its modulating activity in the intermembrane space.

In the yeast *Saccharomyces cerevisiae* two VDAC isoforms have been identified of which only one was proved to form a channel. This VDAC isoform, encoded by the *POR1* gene, is called VDAC1 (or porin1) and its properties are highly conserved among other species (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998). Here, we report that mitochondria of *S. cerevisiae* genetically depleted of the *POR1* gene (Δ*por1* mitochondria) contain the VDAC channel modulating activity in the intermembrane space similar to that observed for wild type mitochondria. In the reconstituted system, the fraction containing the VDAC channel modulating activity (termed here the VDAC channel modulator fraction – MF) isolated from either wild type or Δ*por1* mitochondria induces lower conductance levels of the VDAC channel as well as the TOM complex channel without statistically significant differences. Further, the fraction affects both external NADH transport and preprotein translocation across the outer membrane of wild type and Δ*por1* mitochondria in a way dependent on their energized state. Since quantitative differences are observed in the action one might conclude that the presence of the VDAC channel influences the properties of the intermembrane space protein (s).

MATERIAL AND METHODS

Isolation of Mitochondria and Mitoplasts

A wild type strain of the yeast *Saccharomyces cerevisiae* M3 (*MATa, lys2 his4 trp1 ade2 leu2 ura3*) and VDAC1 (porin1)-depleted mutant M22-2 (Δ*por1*) (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998) were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5. Mitochondria were isolated according to the published procedure (Daum *et al.*, 1982). Mitoplasts were obtained by swelling–shrinking procedure essentially as described in Daum *et al.* (1982). The estimation of the integrity of the outer membrane was based on the permeability of the membrane to exogenous cytochrome *c* (Douce *et al.*, 1984) or on immunodecoration of Western blots with antisera against yeast marker proteins of the intermembrane space (cytochrome c_1 heme lyase) and mitochondrial matrix (Mge 1p). The calculated mean value of the degree of the outer membrane intactness was 96 and 94% for wild type and Δ*por1* mitochondria, respectively.

Isolation of the VDAC Channel Modulating Activity Containing Fraction (the VDAC Channel Modulator Fraction – MF) and Preparation of the Outer Membrane Channels

The intermembrane space of wild type and Δ*por1* mitochondria was prepared according to Martin *et al.* (1998) and fractionated by ammonium sulfate precipitation as described in Liu *et al.* (1994). After resuspension in the MDE buffer containing 10 mM MOPS-KOH, pH 7.2, 1 mM DTT and 0.25 mM EDTA, the collected fractions were tested in the reconstituted system (described below) for the presence of the VDAC channel modulating activity. The active fraction (termed here the VDAC channel modulator fraction – MF) was checked by Western blotting with anti-yeast antisera for the presence of marker proteins of the outer and the inner membrane (Tom40 and ADP/ATP carrier, respectively) as well as of the mitochondrial matrix (Mge 1p) and stored in −80°C. Isolations of the outer membrane of wild type and Δ*por1* mitochondria were performed according to Daum *et al.* (1982). The outer membrane released due to mitoplast preparation was purified on step 15–30–60% sucrose gradient buffered with 10 mM Tris-CI, pH 7.4, and sedimented. The obtained outer membrane pellet was suspended in the solubilisation buffer containing 3% Triton X-100, 10 mM Tris-HCI (pH 7.0) and 1 mM EGTA. The suspension of the outer membrane of wild type mitochondria was then

loaded onto a dry hydroxyapatite/celite column to isolate VDAC channel, as described in De Pinto *et al.* (1987). Before reconstitution the preparations of the VDAC channel and the outer membrane of $\Delta por1$ mitochondria were checked by Western blotting with anti-yeast antisera for the presence of ADP/ATP carrier (the inner membrane), Mge 1p (the mitochondrial matrix), and CC_1HL (the intermembrane space) as well as for VDAC (the VDAC channel preparation) and Tom40 (both types of preparations).

Conductance Measurements in Planar Phospholipid Membranes

The planar phospholipid membrane experiments were performed according to Benz *et al.* (1978). Membranes were formed from 2% (w/v) solution of soybean asolectin dissolved in *n*-decane, across a circular hole (surface area about 0.5 mm^2) in the thin wall of a Teflon chamber separating two compartments (cis–trans) filled with unbuffered 1 M KCI, pH 7.0. The chamber was connected with the recording equipment through calomel half-cells. All preparations described above were added in small aliquots (2–9 uL) to the cis compartment. Cis also refers to the compartment where the voltage was held. The amplified signal was monitored with an oscilloscope and recorded on a strip chart recorder.

Synthesis of DC_1D 101–169

The fusion protein called DC_1D 101–169 consists of amino acid residues 101–169 of the yeast *S. cerevisiae* cytochrome c_1 heme lyase (CC_1HL) comprising the mitochondrial targeting signal and inserted into the middle of dihydrofolate reductase (DHFR) (Diekert *et al.*, 1999). Radiolabelled form of DC_1D 101–169 was synthesized in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S] methionine. (The plasmid containing the fusion protein gene was a kind gift of Professor R. Lill.)

DC_1D 101–169 Translocation by the TOM Complex Channel

Mitochondria were incubated in the import buffer (250 mM sucrose, 20 mM KCI, 10 mM MOPS-KOH, pH 7.2, 5 mM $MgCl_2$, 3% BSA, 0.5% ethanol) for 15 min at 25°C in the presence of [^{35}S] DC_1D 101–169 and 5 μg of MF per 50 μg of mitochondrial protein (Liu and Colombini, 1992) (or a proper volume of MDE buffer). When indicated valinomycin at concentration of

0.16 μg/mg of mitochondrial protein was added to the import buffer. To assay for DC_1D 101–169 translocation across the outer membrane mitochondria were washed with SM buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2), reisolated (10 min, 12,000g) and after resuspension in the same buffer treated with proteinase K (250 μg/mL, 10 min, 0°C). The proteinase K was halted by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). After reisolation (10 min, 12,000g) mitochondria were subjected to SDS-PAGE (Lämmli, 1970). Translocation of DC_1D 101–169 was visualized by fluorography and quantified by ScanPack 3.0.

Determination of the Rate of NADH Oxidation

Mitochondria or mitoplasts were incubated as for the DC_1D 101–169 translocation reaction (see above) in the presence of different external NADH concentrations and 5 μg of MF per 50 μg of mitochondrial protein (Liu and Colombini, 1992). NADH oxidation was monitored spectrophotometrically.

Other Methods

Protein concentrations were measured by the method of Bradford. Respiration of mitochondria and mitoplasts was monitored at 25°C with Rank oxygen electrode in the incubation volume of 0.5 mL. Changes of the inner membrane potential ($\Delta\Psi$) were monitored with tetraphenylophosphonium(TPP^+)-specific electrode as described in Kamo *et al.* (1979). Following SDS-PAGE, the gels were stained with either Coomassie blue or silver (standard methods).

RESULTS AND DISCUSSION

In the yeast *S. cerevisiae* two VDAC isoforms have been identified (VDAC1 and VDAC2) of which only VDAC1 was proved to form a channel (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998). We analyzed channel forming activity of VDAC1 by reconstitution the protein into lipid bilayers. It was achieved by adding its purified preparation to the aqueous phase of one side (so called cis side) of a black membrane bilayer made of asolectin. It is well known that the VDAC channel behavior in planar phospholipid membranes made of asolectin is symmetrical (Colombini, 1994; Colombini *et al.*, 1996). It means that the channel closes at about the same rate and to about the same extent regardless the sign of the applied potential.

The sign is, however, important for the action of the VDAC channel modulator. The modulator acts from both sides of the VDAC channel incorporated into planar membranes provided the modulator-containing side is made negative (Elkeles *et al.*, 1997; Holden and Colombini, 1988, 1993; Liu *et al.*, 1994; Liu and Colombini, 1992). Therefore, in our experiments a membrane potential of −10 mV at cis side was imposed. Taking into account the obtained distribution of conductances (Fig. 1(A)), an average conductance for the VDAC channel of about 4 nS (for 105 single insertions) in 1 M KCl was calculated. The value is consistent with previous studies (Holden and Colombini, 1988; Liu and Colombini, 1992) and corresponds to the dominance of a fully open state (Benz, 1994). The addition of the VDAC channel modulator fraction isolated from either wild type or Δ*por1* mitochondria (MFwt and MFΔ*por1*, respectively) to the cis side at a final protein concentration of approximately 3 μg/mL distinctly changed distribution of conductances of the VDAC channel (Fig. 1(B) and (C)). In both cases an increase of the VDAC channel. lower conductances (corresponding to the dominance of closed states) was observed. Thus, the expression of the intermembrane space protein (s) able to facilitate the VDAC channel closure seems to be independent of the presence or the absence of the channel. In the presence of MFwt (95 single insertions) the calculated average conductance was only slightly lower than that obtained in the presence of MFΔ*por1* (98 single insertions), approximately 2 and 2.5 nS, respectively. Moreover, analysis of

the obtained distributions of conductance values by *t* test (at $t = −0.06946$, $p = 0.94525$ and $\alpha = 0.01$) indicated that the two means were not significantly different. This means that the probability of the VDAC channel transition into low-conductance states is in principle the same in the presence of MFwt and MFΔ*por1*. Thus, under the applied conditions, no difference in MFwt and MFΔ*por1* action on the VDAC channel permeability is observed.

Since the TOM complex channel was reported to contribute to metabolite transport under conditions of genetic or physiological elimination of the VDAC channel functions (Antos *et al.*, 2001a,b; Kmita and Budzińska, 2000) we tested the effect of the obtained VDAC channel modulator fractions on the TOM complex channel permeability in the reconstituted system. So far only two channels have been identified in the outer membrane of *S. cerevisiae* mitochondria, namely the VDAC channel and the TOM complex channel. Thus, we tested whether the purified preparation of the outer membrane of *S. cerevisiae* mitochondria devoid of the VDAC channel (Δ*por1* mitochondria) may be used in the study of the TOM complex channel. For that purpose we analyzed a channel forming activity of the preparations in the reconstituted system. Since it is known that polarity of applied potentials has no influence on the TOM complex channel activity (Athing *et al.*, 2001: Künkele *et al.*, 1998a,b), a negative potential was imposed, which is important for the action of MF (see above). As shown in Fig. 2(A), a histogram of 90 insertion events, recorded in 1 M KCl at membrane potential of
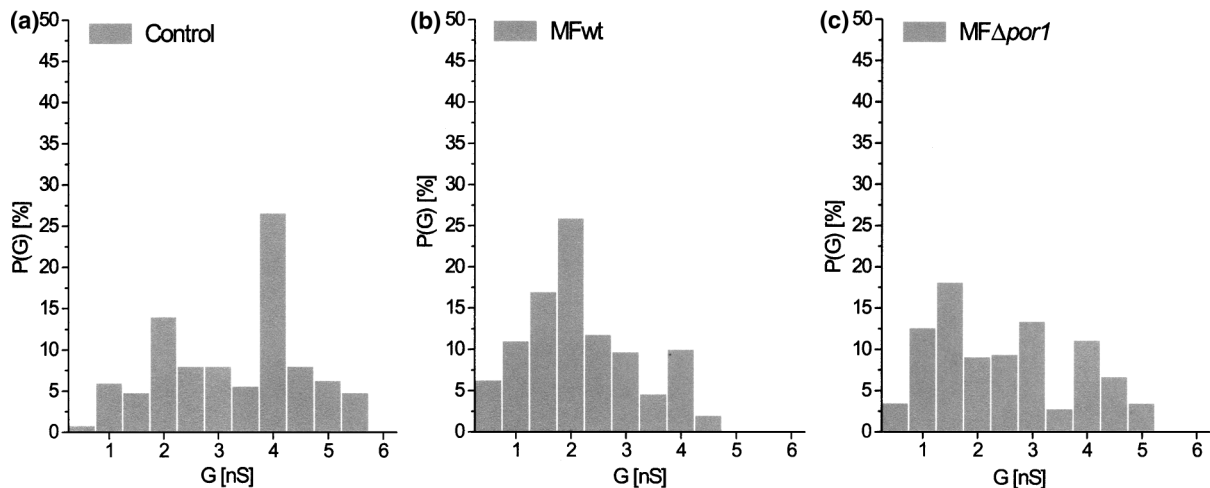

Fig. 1. Histograms of the VDAC channel conductances calculated in the presence of fractions containing the VDAC channel modulating activity isolated from wild type and Δ*por1* mitochondria (MFwt and MFΔ*por1*, respectively). (A) Control measurements in the absence of modulating fractions. (B) The VDAC channel conductances in the presence of MFwt. (C) The VDAC channel conductances in the presence of MFΔ*por1*. In all cases single channel conductances were measured at a membrane potential of −10 mV. The data represent the mean conductances of $n = 105$ (A), 95 (B), and 98 (C). $P(G)$ is the probability that a given conductance increment G is observed.
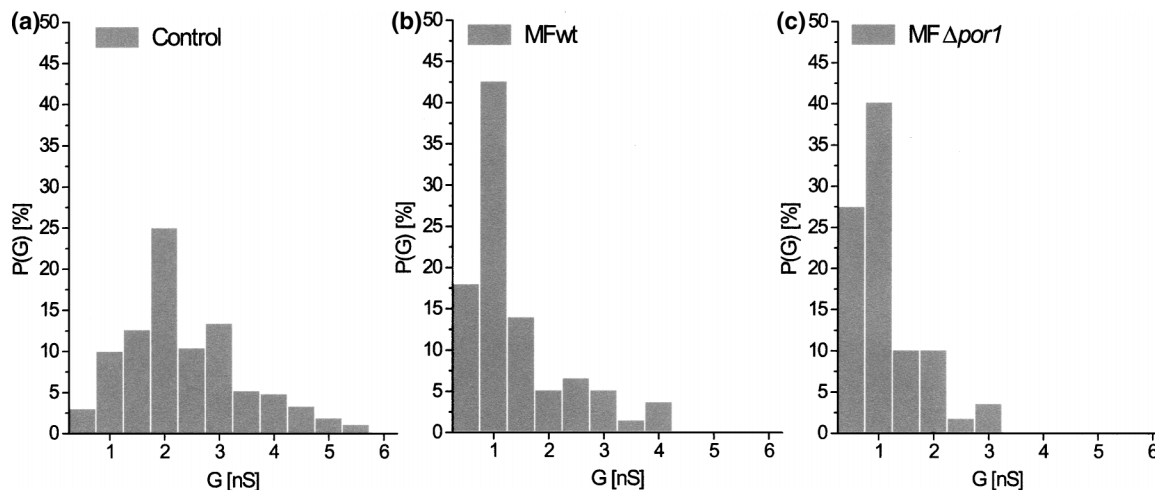
Fig. 2. Histograms of drops in channel conductances recorded for the outer membrane of $\Delta por1$ mitochondria in the presence of fractions containing the VDAC channel modulating activity isolated from wild type and $\Delta por1$ mitochondria (MFwt and MF$\Delta por1$, respectively). (A) Control measurements in the absence of modulating fractions. (B) The conductances in the presence of MFwt. (C) The conductances in the presence of MF$\Delta por1$. In all cases single channel conductances were measured at a membrane potential of -10 mV. The data represent the mean conductances of $n = 90$ (A), 92 (B), and 95 (C). $P(G)$ is the probability that a given conductance increment G is observed.

-10 mV, revealed an average conductance level of about 2.2 nS. This is comparable to those calculated for the TOM holo complex and the TOM core complex isolated from *Neurospora crassa* and corresponds to an open state of the channel (Ahting *et al.*, 1999; Künkele *et al.*, 1998a,b) Further, the fusion protein pb$_2$-DHFR consisting of the first 167 amino acid residues of yeast cytochrome b_2 precursor comprising the mitochondrial targeting sequence and the entire mouse DHFR reduced the observed channel open-state probability in a voltage-dependent manner when added in chemical amounts to the cis site at positive voltages. Control proteins devoid of a mitochondrial presequence had no influence on the channel as well as pb$_2$-DHFR at negative voltages (Ahting *et al.*, 2001; Künkele *et al.*, 1998b) (not shown). Therefore, we used the outer membrane preparation of $\Delta por1$ mitochondria to study the effect of MFwt and MF$\Delta por1$ on the TOM complex channel permeability in the reconstituted system. The addition of the fractions to the cis side (at a final protein concentration of approximately 3 μg/mL) led to distinctly decreased channel conductances that corresponds to the transition from open to closed states (Fig. 2(B) and (C)). In the presence of MFwt (92 single insertions) the calculated average conductance was slightly higher than that obtained in the presence of MF$\Delta por1$ (95 single insertions), approximately 1.4 and 1.2 nS, respectively. When we analyzed the obtained distributions of conductance levels by t test (at $t = -0.05313$, $p = 0.9581$, and $\alpha = 0.01$) the two means turned out to be insignificantly different. Thus, the observed difference is not staistically signifi-

cant, which means that MFwt and MF$\Delta por1$ promote the TOM complex channel closure with the same probability. Since the TOM complex channel is voltage-dependent in reconstituted systems (Ahting *et al.*, 2001; Künkele *et al.*, 1998a,b), it can not be excluded that as in the case of the VDAC channel, the modulation consists in increasing the dependence of the channel on the existing potential. Thus, the voltage gating of the TOM complex channel might be relevant for its physiological role.

According to the published data the VDAC channel modulator incubated with intact mitochondria reduces transport of adenine nucleotides across the outer membrane by inducing VDAC channels to enter closed states as observed in the reconstituted system (Liu and Colombini, 1992). Therefore, we tested whether the obtained fractions were also able to modulate the function of the VDAC channel and the TOM complex channel in intact mitochondria. Since the inner membrane potential may contribute to the existence of a potential across the outer membrane (Benz, 1994; Lemeshko, 2002), experiments were performed with coupled ($+\Delta\Psi$) and uncoupled ($-\Delta\Psi$) mitochondria. The term "coupled mitochondria ($+\Delta\Psi$)" denotes respiration induced by the addition of a respiratory substrate in the absense of ADP (so called "State 2") while the term "uncoupled mitochondria ($-\Delta\Psi$)" corresponds to the respiration but in the presence of uncoupler (valinomycin at concentration of 0.16 μg/mg of mitochondrial protein), It should be, however, remembered that changes in the inner membrane potential trigger changes in mitochondria, which might influence more directly

A. Wild type mitochondria

B. Δpor1 mitochondria.

C. NADH oxidation rate
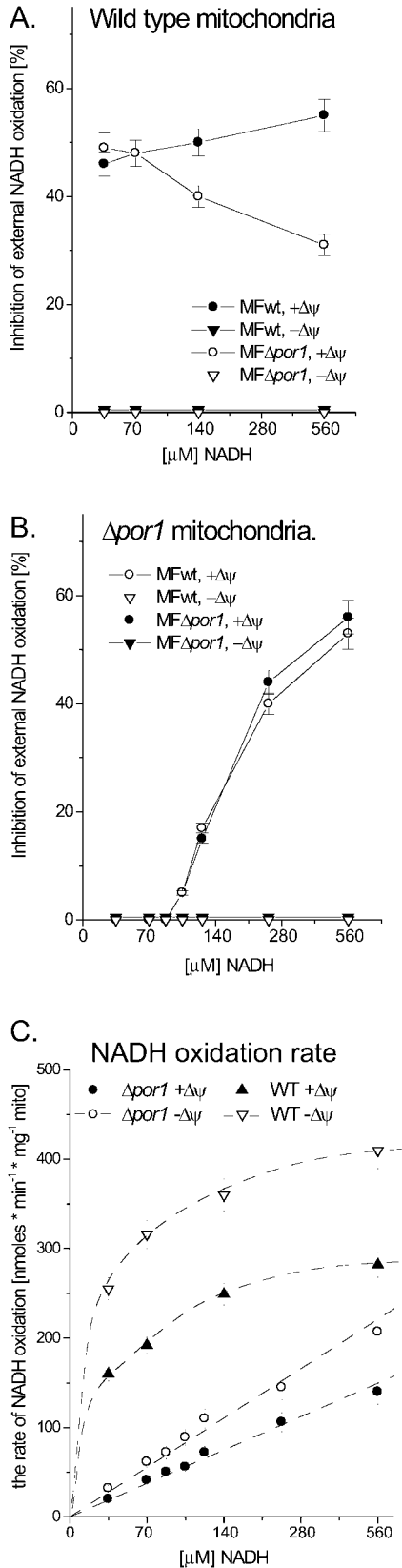
generation of the potential across the outer membrane and activity of the membrane channels. For example coupled and uncoupled mitochondria display different volume of the intermembrane space, which means differences in protein concentration in the compartment leading to differences of a Donnan potential across the outer membrane (Colombini, 1994; Liu and Colombini, 1991, 1992). Thus, using terms "in the presence of the inner membrane potential" or "in the absence of the inner membrane potential" we take into account adequate changes in mitochondria morphology. To study the VDAC channel permeability in intact mitochondria we chose the oxidation of external NADH because it has been shown that (i) VDAC1 (porin1) is crucial to external NADH transport across the outer membrane of *S. cerevisiae* mitochondria (Lee *et al.*, 1998) and (ii) the oxidation of the substrate by the mitochondria enables simple estimation of metabolite passage through the outer membrane (Lee *et al.*, 1998; Michejda *et al.*, 1994), since the substrate is oxidized by the dehydrogenase located on the outer surface of the inner membrane (De Vries and Marres, 1987). As shown in Fig. 3(A), MFwt and MFΔ*por1* had no effect on external NADH oxidation by uncoupled wild type mitochondria, i.e., in the absence of the inner membrane potential ($-\Delta\Psi$). However in the presence of $\Delta\Psi$ ($+\Delta\Psi$), different modes of inhibition of external NADH oxidation by wild type mitochondria were observed, dependent on the source of applied MF. Because lower rates of external NADH oxidation were observed for coupled mitochondria (Fig. 3(C)) one could conclude that the rate of the substrate transport through the outer membrane is not crucial to the action of both MF. Although a fast increase in the inhibition was observed for both MFwt and MFΔ*por1* up to external NADH concentration of approximately 35 μM, in the presence of higher concentrations of the substrate, the fractions imposed different effects on the rate of its oxidation. While a further increase in the inhibition was obtained for MFwt, in

Fig. 3. Inhibition of external NADH oxidation by wild type and Δ*por1* mitochondria imposed by fractions containing the VDAC channel modulating activity isolated from wild type and Δ*por1* mitochondria (MFwt and MFΔ*por1*, respectively). Fifty microgram of mitochondria were incubated in the import buffer in the presence of 5 μg of a given MF (or a proper volume of MDE buffer as the respective control) as described in Material and Methods section. To obtain uncoupled mitochondria valinomycin was used at concentration of 0.16 μg/mg of mitochondrial protein. Different concentrations of external NADH were applied and their oxidation was recorded spectrophotometrically in the presence or absence of the inner membrane potential, $+\Delta\Psi$ or $-\Delta\Psi$, respectively. The levels of inhibition varied by not more than 12.5% in various experiments. Part C shows the rates of external NADH oxidation calculated for coupled ($+\Delta\Psi$) and uncoupled ($-\Delta\Psi$) wild type and mutant mitochondria incubated in the absence of MF.

the presence of MFΔpor1, the inhibition decreased progressively. Thus, in the case of wild type mitochondria, MFwt and MFΔpor1 display a quantitative difference in modulating activity, which might result from the presence and the absence of the VDAC channel in source mitochondria. The difference was not observed in the case of the reconstituted VDAC channel and might be triggered by the presence of other mitochondiral protein (s) absent from the reconstituted system. On the other hand, it is also possible that pleiotropic consequences of yeast cells and mitochondria lacking the VDAC channel might be responsible for changes in the intermembrane space protein properties. This, however, would be evoked by the absence of the VDAC channel. Thus, the relation between the presence of the VDAC channel and properties of the intermembrane space proteins seems to exist.

Since it is know that the TOM complex channel may serve as an emergency pathway for external NADH across the outer membrane of *S. cerevisiae* mitochondria (Antos *et al.*, 2000 a,b; Kmita and Budzińska, 2000), the effect of MFwt and MFΔpor1 on external NADH oxidation by Δpor1 mitochondria was also tested. As shown in Fig. 3(B), both fractions caused the same levels of inhibition of external NADH oxidation by Δpor1 mitochondria but again only in the case of coupled mitochondria although the rate of the substrate oxidation was higher under conditions of uncoupling (Fig. 3(C)). Thus, independently of the presence of the VDAC channel, the inhibition of external NADH oxidation imposed by MFwt and MFΔpor1 occurs only in the presence of the inner membrane potential. However, in the case of Δpor1 mitochondria higher concentrations of external NADH (above 88 μM) are required for the inhibition to proceed. It has been suggested (Rostovtseva *et al.*, 2002) that interaction of NADH with binding sites within the VDAC channel enhances NADH flux at its low concentrations and slows the flux down at its high concentrations. The same might be true for the TOM complex channel although the selectivity of NADH binding sites should be lower since the metabolite transport through the outer membrane of Δpor1 mitochondria is strongly restricted (Kmita *et al.*, 1999; Lee *et al.*, 1998). Thus, changes of the outer membrane channel permeability imposed by NADH concentrations might add to or be enhanced by MF acting in a way dependent on the energized state of mitochondria. Since the same effect of the presence of MFwt and MFΔpor1 is observed with Δpor1 mitochondria, the differences in their influence on external NADH oxidation by wild type mitochondria result probably from MF action on the VDAC channel permeabillity. It should be emphasized here that the inhibitory effect of MFwt and MFΔpor1 on external NADH oxidation disappeared in mitoplasts, i.e., after the removal of the outer membrane. Besides, the fractions at the concentration applied had no direct effect on the NADH concentrations and did not display a NAD^+ reducing activity as tested spectrophotometrically (not shown).

To check whether MFwt and MFΔpor1 have indeed the effect on the TOM complex channel permeability in intact mitochondria we studied their influence on preprotein translocation across the outer membrane of wild type and Δpor1 mitochondria. For that purpose a fusion protein termed DC_1D101–169 (Diekert *et al.*, 1999) was chosen. Translocation of the protein into the intermembrane space, such as native heme lyases, occurs independently of the inner membrane potential and does not require ATP (Diekert et al., 1999; Steiner *et al.*, 1995). This makes possible to study the effect of the presence of the inner membrane potential on the TOM channel permeabillity and ignore ATP contribution to the translocation efficiency. Taking into account that external NADH may be transported by the TOM complex channel, ethanol, diffusing freely through lipid membranes, was applied as a respiratory substrate. The total amount of $[^{35}S]$ DC_1D 101–169 translocated by the TOM complex channel was determined by quantification of bands representing $[^{35}S]$ DC_1D 101–169 fraction protected against externally added proteinase K (Fig. 4(C)). Thus, the fraction corresponds to $[^{35}S]$ DC_1D 101–169 fully imported into the intermembrane space. As shown in Fig. 4(A) and (B), independently of the type of studied mitochondria, MFwt and MFΔpor1 affected $[^{35}S]$ DC_1D101–169 translocation in a similar way. When compared with control samples incubated in the presence of a proper volume of the applied buffer, MF caused an increase in the preprotein translocation both in coupled ($+\Delta\Psi$) and uncoupled ($-\Delta\Psi$) mitochondria (Fig. 4(C), $-$MF and $+$MF, respectively). This suggests that the mode of MF action does not consist in the blockage of the TOM complex channel. The increase was higher in Δpor1 mitochondria, independently of the MF applied, probably due to the upregulation of the TOM complex components (Antos *et al.*, 2001a,b; Kmita and Budzińska, 2000) enabling higher levels of $[^{35}S]$ DC_1D 101–169 translocation across the outer membrane. However, with a given type of mitochondria, the increase in $[^{35}S]$ DC_1D 101–169 translocation caused by MFΔpor1 was lower than that observed for MFwt. Moreover, the effect of MFwt and MFΔpor1 was pronounced more clearly in the case of uncoupled mitochondria and the differences observed between coupled and uncoupled mitochondria were comparable for both types of the studied mitochondria. Therefore, it might be suggested that obtained fractions contain competing the TOM complex channel modulating activities, namely decreasing the channel permeability in the presence of the inner membrane potential
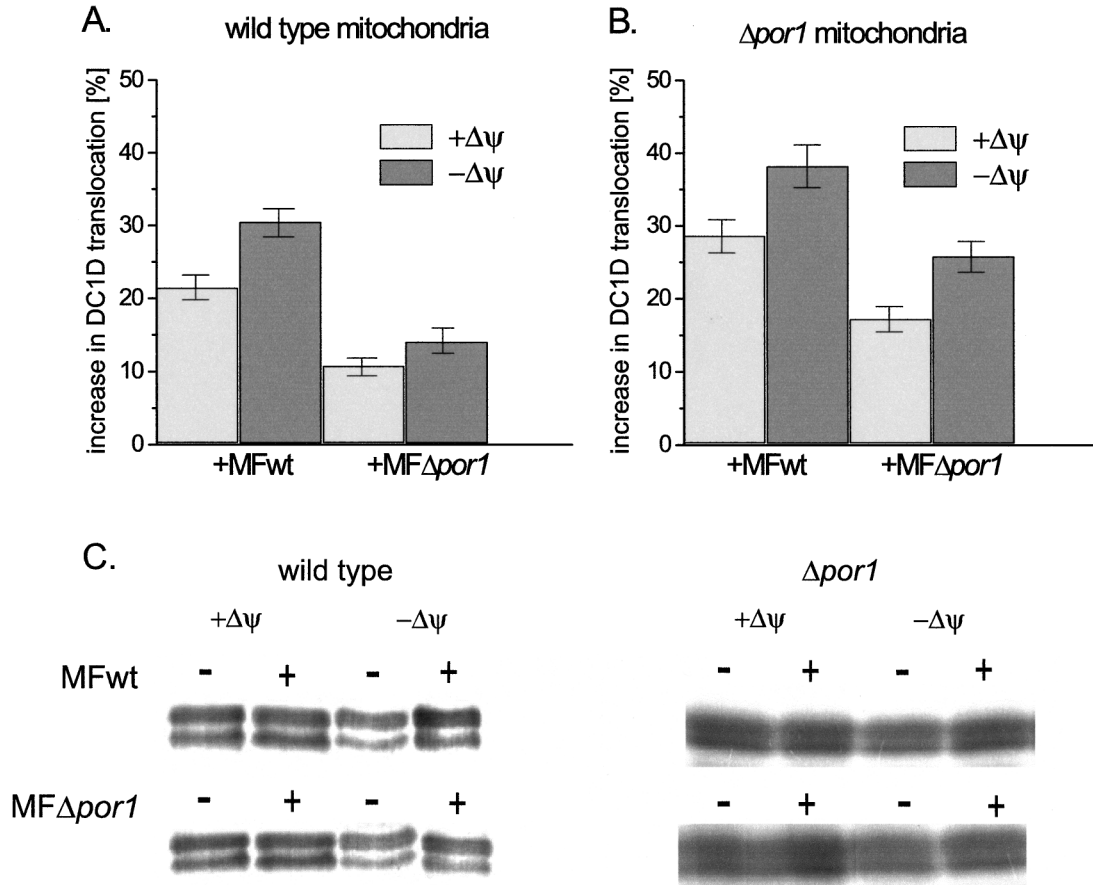
Fig. 4. Increase in [^{35}S] DC$_1$D 101–169 translocation across the outer membrane of wild type and $\Delta por1$ mitochondria in the presence of fractions containg the VDAC channel modulating activity isolated from wild type and $\Delta por1$ mitochondria (MFwt and MF$\Delta por1$, respectively). Coupled ($+\Delta\Psi$) and uncoupled ($-\Delta\Psi$) mitochondria were incubated in the import buffer in the presence of [^{35}S] DC$_1$D 101–169 and 5 μg of MFwt or MF$\Delta por1$ per 50 μg of mitochondrial protein (or a proper volume of MDE buffer as the respective control) as described in Material and Methods section. To obtain uncoupled mitochondria valinomycin was applied at concetration of 0.16 μg/mg of mitochondrial protein. After incubation samples were washed with SM buffer and reisolated (10 min, 12,000g). The pellets were resuspended in SM buffer and treated with proteinase K (250 μg/mL, 10 min, 0°C) halted by 1 mM PMSF. After reisolation (10 min, 12,000g) samples were subjected to SDS-PAGE. Bands representing [^{35}S] DC$_1$D 101–169 translocated across the outer membrane were visualized by fluorography and quantified by ScanPack 3.0. The obtained data varied by not more than 12% in various experiments. (C) Typical results of experiments used to calculate the differences in DC$_1$D 101–169 translocation shown in (A) (wild type mitochondria) and (B) ($\Delta por1$ mitochondria).

and enhancing preprotein translocation in the absence of the potential. In spite of the same mode of action, MFwt and MF$\Delta por1$ display different levels of the modulating capability, which points again to a quantiative difference between action of MF isolated from wild type and $\Delta por1$ mitochondria on the both types of intact mitochondria (see above).

The difference might result from a changed pattern of expression of the intermembrane space proteins occurred in the absence of the VDAC channel. It seems to be confirmed by the upregulation of the TOM complex components observed in the absence of the VDAC channel (Kmita and Budzińska, 2000). Therefore, we an-

alyzed both fractions by SDS-PAGE. As shown in Fig. 5, nearly all bands present in MFwt were also observed for MF$\Delta por1$, although some of them in smaller amounts. The only band missing from MF$\Delta por1$ corresponded to an approximately 35 kD protein (marked with the arrow), not reported previously as being important for the VDAC channel modulating activities (Liu *et al.*, 1994; Liu and Colombini, 1991). The protein is rather not obligatory for the ability of MFwt to decrease the VDAC channel conductance since there is no difference between MFwt and MF$\Delta por1$ activities in the reconstituted system and only a quantitative difference between their effects in the case of intact wild type mitochondria. However, the identity
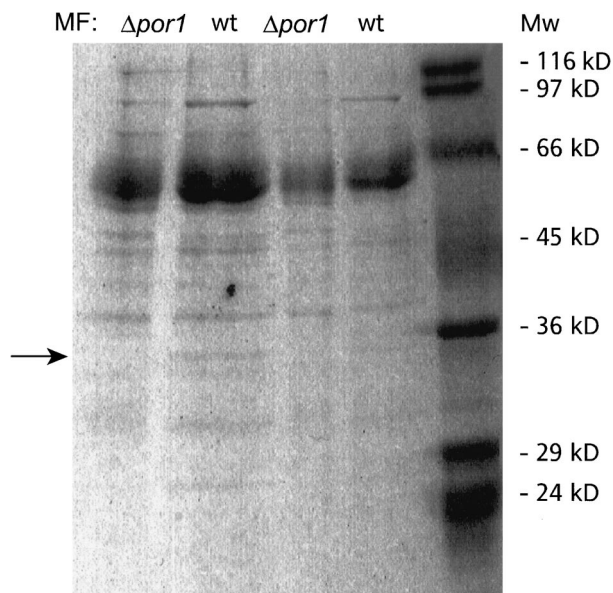
Fig. 5. SDS-polyacrylamide gel electrophoresis (12%) of the VDAC channel modulator fractions isolated from either wild type or Δ*por1* mitochondria (MFwt and MFΔ*por1*, respectively). Proteins were visualized by silver staining. The figure presents typical results obtained with about 5 and 10 μg of the studied fractions.

and physiological role of the 35 kD protein should be addressed. The research is under way.

Summing up, the obtained results confirm the existence of the VDAC channel modulating activity in the intermembrane space of *S. cerevisiae* mitochondria (Holden and Colombini, 1988) and indicate that the activity is also present in the yeast mitochondria devoid of the VDAC channel. Further, the fraction of the intermembrane space proteins containing the VDAC channel modulating activity is also able to modulate the TOM complex channel. Thus, further studies are required to identify the responsible protein(s) to exclude the possibility that the observed modulating activities result from low specificity of involved protein(s) simultaneously affecting the functional state of both types of the outer membrane channels. Nevertheless, the finding that both modulating activities are influenced by the presence of the inner membrane potential indicates that the energized state of mitochondria might play an important role in regulation of the outer membrane permeability. However, it should be remembered that the studied modulating protein(s) are located in the intermembrane space so their ability to act on the outer membrane channels of native mitochondria from cytoplasmic side might result from the presence of a similar modulating protein(s) in cytoplasmic compartment (Liu *et al.*, 1994). Our preliminary results seem to confirm the

possibility. So it is reasonable to conclude that the intermembrane space proteins constitute only a part of cellular mechanism controlling the outer membrane channel permeability.

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