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The TOM complex is involved in the release of superoxide anion from mitochondria

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Abstract Available data indicate that superoxide anion ($O_2^{\bullet-}$) is released from mitochondria, but apart from VDAC (voltage dependent anion channel), the proteins involved in its transport across the mitochondrial outer membrane still remain elusive. Using mitochondria of the yeast *Saccharomyces cerevisiae* mutant depleted of VDAC (Δ*por1* mutant) and the isogenic wild type, we studied the role of the TOM complex (translocase of the outer membrane) in the efflux of $O_2^{\bullet-}$ from the mitochondria. We found that blocking the TOM complex with the fusion protein pb_2-DHFR decreased $O_2^{\bullet-}$ release, particularly in the case of Δ*por1* mitochondria. We also observed that the effect of the TOM complex blockage on $O_2^{\bullet-}$ release from mitochondria coincided with the levels of $O_2^{\bullet-}$ release as well as with levels of Tom40 expression in the mitochondria. Thus, we conclude that the TOM complex participates in $O_2^{\bullet-}$ release from mitochondria.

Keywords *Saccharomyces cerevisiae* · TOM complex · Superoxide anion

Introduction

Tight coordination between the nucleus and mitochondria is required for maintenance of mitochondrial function and includes both anterograde (nucleus to mitochondria) and retrograde (mitochondria to nucleus) signals (e.g. Allen 2003; Butow and Avadhani 2004; Pesaresi et al. 2007; Woodson and Chory 2008). Anterograde mechanisms coordinate gene expression in mitochondria in response to endogenous and environmental signals that are perceived by the nucleus, whereas retrograde mechanisms transmit signals that originate in mitochondria to regulate nuclear gene expression, which can then alter anterograde control. Signals relevant to retrograde mechanisms can involve reactive oxygen species (ROS) generated and released by mitochondria, which contribute to intracellular redox homeostasis and to regulation of signaling cascades (Finkel 2003; Han et al. 2003; Liu et al. 2005; Storz 2006; Budzinska et al. 2007). However, when such ROS release evades or overcomes cell defenses, it can damage a wide range of macromolecules in the cell, including nucleic acids, proteins and lipids, eventually leading to cell dysfunction and death (Madesh and Hajnóczky 2001; Duchen 2004; Storz 2006; Monsalve et al. 2007).

The best known ROS originating from mitochondria are hydrogen peroxide (H_2O_2) and its stoichiometric precursor superoxide anion ($O_2^{\bullet-}$) (Han et al. 2001; Jezek and Hlavatá 2005). They are generated by the mitochondrial respiratory chain as by-products of cellular energy production. From the sites of generation, $O_2^{\bullet-}$ is released into the mitochondrial matrix and into the intermembrane space, where it can be dismutated by proper dismutases to molecular oxygen and H_2O_2. The latter can be converted to water by other antioxidant enzymes or to hydroxyl radical in the presence of some transition metals (e.g. Stohs and Bagchi 1995; Sturtz et al. 2001; St-Pierre et al. 2002; Jezek and Hlavatá 2005; Storz 2006; Culotta et al. 2006). It has been shown that H_2O_2 diffuses rapidly through membranes (Antunes and Cadenas 2000), and the release of H_2O_2 from mitochondria to the cytosol reflects

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the balance between its production and consumption reactions (Han et al. 2003). However, $O_2^{\bullet-}$ is generally considered to be membrane-impermeable, except for the small fraction in protonated perhydroxyl radical form (Gus'kova et al. 1984; Han et al. 2001). Thus, the question arises as to how $O_2^{\bullet-}$ vectorially released into the intermembrane space can diffuse into the cytosol across the outer membrane of mitochondria.

It was shown by Han et al. (2003) that $O_2^{\bullet-}$ exited mitochondria via VDAC (voltage dependent anion channel), known also as mitochondrial porin (for reviews see, for example, Benz 1994; Blachly-Dyson and Forte 2001; DePinto et al. 2003; Colombini 2004; Shoshan-Barmatz et al. 2008; Mannella and Kinnally 2008). Physiologically, VDAC functions as a major channel allowing passage of metabolites between the intermembrane space of mitochondria and the cytoplasm. However, Han et al. (2003) also suggested that $O_2^{\bullet-}$ may diffuse through channels in the outer membrane other than VDAC. Accordingly, we observed that mitochondria of the yeast *Saccharomyces cerevisiae* mutant depleted of VDAC (Δ*por1* mutant) were still able to release $O_2^{\bullet-}$ (Budzinska et al. 2007). Beside VDAC, there are two other channels in the mitochondrial outer membrane of *S. cerevisiae*. They belong to the protein import machinery, and are components of the TOM complex (translocase of the mitochondrial outer membrane) and the TOB/SAM complex (topogenesis of the mitochondrial outer membrane β-barrel proteins/sorting and assembly machinery) (for reviews see, for example, Dolezal et al. 2006; Bohnert et al. 2007; Becker et al. 2008; Mokranjac and Neupert 2009). Subunits of the TOM complex, i.e. Tom proteins are upregulated in mitochondria of Δ*por1* mutant of *S. cerevisiae* (Kmita and Budzińska 2000; Galganska et al. 2008) and under certain conditions Δ*por1* mitochondria release more $O_2^{\bullet-}$ than those of the isogenic wild type (Budzinska et al. 2007). Therefore one might speculate that the TOM complex contributes to efflux of $O_2^{\bullet-}$ from the mitochondria.

It is believed that $O_2^{\bullet-}$ exits mitochondria via membrane anion channels (Zuo et al. 2003; Han et al. 2003). The TOM complex contains a channel with a selectivity for cations (Hill et al. 1998; Künkele et al. 1998), but anions may also move within the channel (Wojtkowska et al. 2005). Therefore, we evaluated the effect of blocking the TOM complex on $O_2^{\bullet-}$ release from Δ*por1* and wild type *S. cerevisiae* mitochondria. We found that the blockage decreased $O_2^{\bullet-}$ release and the effect was more pronounced for Δ*por1* mitochondria. We also revealed that the effect of the TOM complex blockage on $O_2^{\bullet-}$ release coincided with the levels of $O_2^{\bullet-}$ release as well as with levels of Tom40 expression in the mitochondria. Thus, $O_2^{\bullet-}$ release from mitochondria may occur via the TOM complex.

Material and methods

Yeast strains, culture conditions, isolation of mitochondria and formation of mitoplasts The following *Saccharomyces cerevisiae* strains were studied: the isogenic wild type M3 (*MAT*a, *lys2 his4 trp1 ade2 leu2 ura3*) and VDAC1 (porin1)-depleted mutant M22-2 (Δ*por1*) (Blachly-Dyson et al. 1997; Lee et al. 1998). Yeast cells were grown at 28ºC in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5 to exponential phase (OD_{546} of 0.9–1.1) or to stationary phase (OD_{546} of 2.0–2.3). For modification of exponential phase, 10 μM menadione (wild type) or 10 mM ascorbate (Δ*por1*) was added to a given cell culture at 0.1 OD and the cells were grown until standard exponential phase (Galganska et al. 2008). Mitochondria were isolated according to the published procedure (Daum et al. 1982), and mitoplasts were obtained by the swelling procedure (Daum et al. 1982). The swelling buffer contained 20 mM Hepes pH 6.9 and 0.2% bovine serum albumin (BSA). The estimation of the integrity of the outer membrane was based on the permeability of the membrane to the exogenous cytochrome c (Douce et al. 1984). The calculated mean value of the mitochondrial outer membrane intactness were 95% and 93% for wild type and Δ*por1* mitochondria, respectively.

Determination of $O_2^{\bullet-}$ release from mitochondria and mitoplasts The levels of $O_2^{\bullet-}$ release from mitochondria and mitoplasts were determined by measuring the superoxide dismutase-inhibitable reduction of nitroblue tetrazolium (NBT) (Green and Hill, 1984). NBT was added to a final concentration of 0.07 mg/ml and its reduction rate was measured at 560 nm (UV 1602, Shimadzu). Mitochondria (native or trypsin-pretreated) or mitoplasts incubated with pb_2-DHFR (or with an adequate volume of pb_2-DHFR buffer instead of the protein in control samples) as for the TOM complex blockage (see below) were incubated in M buffer (0.65 M mannitol, 10 mM Hepes pH 6.9, 10 mM phosphate buffer pH 6.9, 5 mM KCl, 4 mM $MgCl_2$, 0.2% BSA) in the presence of 30 mM ethanol, which was added before NBT. The calculated values of $O_2^{\bullet-}$ release from the studied mitochondria or mitoplasts (in pmol/min/mg protein) were tested by *t* test ($\alpha = 0.01$) and they turned out to be significantly different.

TOM complex blockage by pb_2-DHFR The fusion protein pb_2-DHFR consists of the first 167 amino acid residues of the yeast cytochrome b_2 precursor including the mitochondrial targeting sequence with the intermembrane space sorting signal (pb_2) and the entire mouse dihydrofolate reductase (DHFR) sequence. The high level expression and purification of pb_2-DHFR was performed as described by Wienhues et al. (1992). The TOM complex blockage by pb_2-DHFR was performed essentially as described in Antos et al. (2001b). For the import reaction ("import"), mito-

chondria or mitoplasts were incubated in the import buffer (250 mM sucrose, 20 mM KCl, 10 mM MOPS-KOH pH 7, 2, 5 mM $MgCl_2$, 3% BSA, 30 mM ethanol) for 20 minutes at 25ºC in the presence of 1 μg of pb_2-DHFR per 50 μg of mitochondrial protein (if not indicated otherwise). For accumulation of pb_2-DHFR within both mitochondrial membranes ("import+MTX"), pb_2-DHFR was preincubated for 15 min on ice in the presence of 2 μM methotrexate (MTX), which stabilized the folding state of DHFR, and was then applied for the import reaction. To assay for pb_2-DHFR arrest at the level of the outer membrane ("the *trans* side binding"), 0.16 μg of valinomycin and 6 μg oligomycin per mg of mitochondrial protein were added to the import buffer. After incubation mitochondria (or mitoplasts) were washed with HS (High Salt) buffer (250 mM sucrose, 10 mM MOPS-KOH pH 7.2, 120 mM KCl) to remove pb_2-DHFR bound at the surface and reisolated (10 min., 12000 x g). When indicated, after the washing mitochondria (or mitoplasts) were resuspended in SM buffer (250 mM sucrose, 10 mM MOPS-KOH pH7.2) and treated with proteinase K (250 μg/ml, 10 min at 0ºC), which was halted by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). In experiments using trypsin pretreatment, mitochondria were incubated in the presence of the protease (50 μg/ml) for 15 min on ice and then soybean trypsin inhibitor was added (1 mg/ml) prior to the import reaction. In control samples (-trypsin), the protease and its inhibitor were applied simultaneously. After reisolation (10 min, 12 000 x g), the organelles were subjected to SDS-PAGE and pb_2-DHFR was visualized by the ECL methods (Amersham) following immunodecoration with anti-mouse DHFR antiserum (standard procedure) and quantified by ScanPack 3.0.

Other methods Protein concentrations were determined by the Bradford method. Respiration of mitochondria was monitored at 25ºC with a Rank oxygen electrode in an incubation volume of 0.5 ml. The levels of Tom40 and Mge were visualized by the ECL method following immunodecoration with anti-yeast proper antisera and quantified by ScanPack 3.0.

Results and discussion

Levels of $O_2^{\bullet-}$ release from the studied mitochondria coincide with Tom40 expression levels

It is well known that VDAC may be present as isoforms encoded by separated genes, displaying different channel-forming activities and probably playing different roles. The yeast *Saccharomyces cerevisiae* mitochondria express two VDAC isoforms, of which only one has been proven to form a channel (Blachly-Dyson et al. 1997; Lee et al. 1998). This isoform, encoded by the *POR1* gene, is called VDAC1 (or porin 1) and its properties are highly conserved among other species. We have previously shown that in mitochondria of *S. cerevisiae* mutant depleted of VDAC1, namely Δ*por1* mutant, the TOM complex subunits, i.e. Tom proteins are upregulated (Antos et al. 2001a). On the other hand, we have also reported that expression levels of Tom proteins in both Δ*por1* and the isogenic wild type *S. cerevisiae* mitochondria are mediated by the reduction/oxidation (redox) state of the cytosol and increase under more oxidative conditions (Budzinska et al. 2007; Galganska et al. 2008). In the case of Δ*por1* cells the oxidation occurs during exponential growth phase whereas in the case of wild type cells it occurs when the cells shift from exponential to stationary growth phase. The expression levels of Tom proteins in both Δ*por1* and wild type mitochondria coincide with these oxidation events, peaking in exponential growth phase and decreasing distinctly in stationary growth phase for Δ*por1* mitochondria and increasing in stationary growth phase for wild type mitochondria (Galganska et al. 2008). Interestingly, wild type mitochondria release higher amounts of superoxide anion ($O_2^{\bullet-}$) in stationary growth phase, whereas Δ*por1* mitochondria in exponential growth phase (Budzinska et al. 2007). Thus, for the studied *S. cerevisiae* strains, changes in Tom protein levels in mitochondria seem to coincide with levels of $O_2^{\bullet-}$ release from the mitochondria.

Here, we also studied the mitochondria isolated from cells in modified exponential growth phase. We have observed for the studied *S. cerevisiae* strains that modification of exponential growth phase toward stationary growth phase with regard to the cytosol redox state results in expression levels of Tom proteins in mitochondria that normally occur in stationary growth phase (Galganska et al., 2008). To obtain the modified exponential growth phase we added an oxidant (10 μM menadione) or an antioxidant (10 mM ascorbate) to the culture medium, depending on the strain as described in "Materials and methods". If the TOM complex participates in $O_2^{\bullet-}$ release from mitochondria, changes in Tom protein expression levels in mitochondria should coincide with changes of $O_2^{\bullet-}$ release from the mitochondria. Thus, modification of exponential growth phase towards stationary growth phase should result in mitochondria releasing the levels of $O_2^{\bullet-}$ usually observed in stationary growth phase.

As shown in Table 1, the calculated values of $O_2^{\bullet-}$ release from mitochondria isolated from cells in stationary and modified exponential growth phases were comparable and differed from those calculated for mitochondria isolated from cells in exponential growth phase. When the levels of $O_2^{\bullet-}$ release from mitochondria were compared between stationary and exponential growth phases as well as between modified exponential and exponential growth phases for a

Table 1 Levels of $O_2^{\bullet-}$ release, the inhibition of $O_2^{\bullet-}$ release by the TOM complex blockage and levels of Tom40 expression for wild type and *Δpor1* mitochondria isolated from cells in exponential, stationary and modified exponential growth phases

	Wild type			*Δpor1*		
Growth phase	Exponential	Stationary	Modified exponential	Exponential	Stationary	Modified exponential
$O_2^{\bullet-}$ release [pmol/min/mg mito protein]						
resting state	6.8±0.8	109.2±14.2	96.7±8.7	248.8±34.8	7.8±0.7	23.4±3.3
uncoupled state	5.8±0.7	93.9±13.1	82.2±10.7	213.9±27.8	6.9±0.9	20.6±1.9
inhibition of $O_2^{\bullet-}$ release [%]						
"import"	2±0.23	4±0.48	8±1.23	16±1.44	10±1.34	10±0.92
"import+MTX"	8±1.12	15±2.11	16±2.28	30±2.12	18±1.98	17±1.87
"the trans site binding"	5±0.65	8±0.63	12±1.67	17±2.38	13±1.77	14±1.32
levels of Tom40 in mitochondria [a.u.]						
	148±18	196±23	215±27	225±29	279±37	254±31

The uncoupled state was obtained by adding of 0.16 μg of valinomycin per mg of mitochondrial protein. The levels of $O_2^{\bullet-}$ release from mitochondria were determined by measuring the superoxide dismutase inhibitable reduction of nitroblue tetrazolium (NBT) as described in "Materials and methods". The TOM complex blockage under "import", "import+MTX" and "the *trans* site binding" conditions was performed as described in "Materials and methods". Next, mitochondria were washed with HS buffer and $O_2^{\bullet-}$ release was measured. Levels of Tom40 expression in mitochondria subjected to SDS-PAGE were visualized by immunodecoration with anti-Tom40 antiserum and quantified by ScanPack 3.0. Data are mean values±SEM of four independent experiments performed for mitochondria isolated from cells in a given growth phase

given type of mitochondria, an increase was observed for wild type mitochondria, whereas a strong decrease was observed for *Δpor1* mitochondria. The calculated values of levels of $O_2^{\bullet-}$ release changed as follows (in pmol/min/mg protein): for wild type mitochondria, 6.8 ± 0.8 (exponential growth phase), 109.2 ± 14.2 (stationary growth phase), 96.7 ± 8.7 (modified exponential growth phase), and for *Δpor1* mitochondria, 248.8 ± 34.8 (exponential growth phase), 7.8 ± 0.7 (stationary growth phase), 23.4 ± 3.3 (modified exponential growth phase). Interestingly, for wild type mitochondria the observed increase in $O_2^{\bullet-}$ release coincided with a distinct increase in expression levels of Tom40, a crucial subunit of the TOM complex (Table 1; at the basis of Fig. 1), whereas for *Δpor1* mitochondria a distinct decrease in $O_2^{\bullet-}$ release was accompanied by only a slight increase in Tom40 levels. When cells shifted from exponential to stationary or to modified exponential growth phase, the calculated values of Tom40 levels in mitochondria changed as follows (in arbitrary units): from 148 ± 18 to 196 ± 23 or to 215 ± 27, respectively, for wild type, and from 225 ± 29 to 279 ± 37 or to 254 ± 31, respectively, for *Δpor1*. Thus, a correlation between $O_2^{\bullet-}$ release and Tom40 expression levels was observed for wild type and *Δpor*1 mitochondria that suggests an involvement of the TOM complex in $O_2^{\bullet-}$ release from both types of the studied mitochondria. Accordingly, it has been suggested by Han et al. (2003) that the TOM complex may transport $O_2^{\bullet-}$ across the outer membrane of mitochondria. To test this

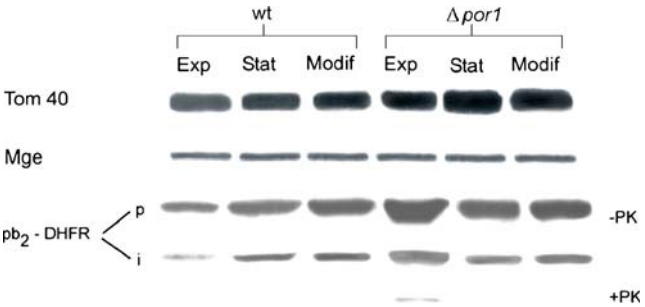

Fig. 1 Correlation between levels of Tom40 expression and pb_2-DHFR arrested under "import+MTX" conditions for wild type and *Δpor1* mitochondria isolated from cells in exponential, stationary and modified exponential growth phases**.** Mitochondria were subjected to SDS-PAGE and levels of Tom40, Mge and pb_2-DHFR arrested under "import + MTX" conditions were visualized by immunodecoration with the proper antisera. Mge serves as a loading control. The figure presents typical results of a Western blot applied to the analysis and obtained with 50 μg of the studied mitochondria. The linear range of Western blot analysis for the studied proteins was tested as described by Kmita et al. (2004). The binding of pb_2-DHFR under "import + MTX" conditions was performed as in "Materials and methods". After incubation mitochondria were resuspended in SM buffer and the samples were divided in two. One aliquot was treated with proteinase K (250 μg/ml, 10 min at 0ºC) (+PK) while the second was left untreated (-PK). Then, mitochondria were subjected to SDS-PAGE. The levels of Tom40, Mge and pb_2-DHFR varied by no more than 15% in the performed experiments (four independent experiments for mitochondria isolated from cells in a given growth phase). p and i denote precursor and intermediate forms of pb_2-DHFR, respectively

hypothesis we blocked the TOM complex in the studied *S. cerevisiae* mitochondria with the fusion protein pb_2-DHFR (1 μg per 50 μg of mitochondrial protein) and checked whether $O_2^{\bullet-}$ release from the mitochondria was affected.

Blocking the TOM complex results in inhibition of $O_2^{\bullet-}$ release from the studied mitochondria and the most effective blockage was obtained under "import+MTX" conditions

The fusion protein pb_2-DHFR is efficiently imported into the studied mitochondria (Antos et al., 2001b) and consists of the first 167 amino acids of yeast cytochrome b_2 preprotein (pb_2) and the entire mouse dihydrofolate reductase (DHFR). We blocked the TOM complex as previously described (Antos et al. 2001b) under the following conditions: (1) "import", (2) "import+MTX" and (3) "the *trans* site binding". Thus, we attained: (1) transient blockage of the TOM complex by imported pb_2-DHFR, (2) stable blockage of the TOM complex due to stabilization of the folded state of DHFR and subsequent arrest of pb_2-DHFR within translocation contact sites involving the TOM and the TIM complexes and (3) stable blockage of the TOM complex by arresting of pb_2-DHFR at the level of the mitochondrial outer membrane due to presequence translocation and binding to the TOM complex at the *trans* site of the complex facing the intermembrane space of mitochondria (Antos et al. 2001b).

We performed the blockage for wild type and Δ*por1* mitochondria isolated from cells in exponential, stationary and modified exponential growth phases and measured the effect of blocking the TOM complex on $O_2^{\bullet-}$ release. The effect was expressed as a percentage of inhibition calculated with respect to control samples containing an adequate volume of pb_2-DHFR buffer instead of the protein. As shown in Table 1, all applied conditions of the TOM complex blockage inhibited $O_2^{\bullet-}$ release from the studied mitochondria, particularly from Δ*por1* mitochondria. It is noteworthy that independently of the TOM complex blockage conditions, the calculated levels of inhibition correlated with changes in Tom40 expression levels in the studied mitochondria (Table 1, Fig. 1). Thus, expression levels of Tom proteins in mitochondria seem to be important for the inhibition of $O_2^{\bullet-}$ release caused by the TOM complex blockage. The least effective blockage was observed under "import" conditions that could result from only transient effect of pb_2-DHFR. When cells shifted from exponential to stationary or to modified exponential growth phase, the calculated levels of inhibition changed as follows (in %): from 2 ± 0.23 to 4 ± 0.48 or to 8 ± 1.23, respectively, for wild type mitochondria and from 16 ± 1.44 to 10 ± 1.34 or to 10 ± 0.92, respectively, for Δ*por1* mitochondria. Only a slightly stronger effect was observed for "the *trans* site binding" conditions as the calculated levels of inhibition changed as follows (in %): for wild type mitochondria, from 5 ± 0.65% (exponential growth phase) to 8 ± 0.63% (stationary growth phase) or to 12 ± 1.67% (modified exponential growth phase) and for Δ*por1* mitochondria, from 17 ± 2.38% (exponential growth phase) to 13 ± 1.77% (stationary growth phase) or to 14 ± 1.32% (modified exponential growth phase). This may result from a decrease in $O_2^{\bullet-}$ release caused by the uncoupler (e.g. Jezek and Hlavata 2005) used for "the *trans* site binding" conditions, although the decrease was rather moderate (Table 1). The most effective inhibition of $O_2^{\bullet-}$ release was obtained under stable blockage of the TOM complex during the resting state ("import+MTX"). When cells shifted from exponential to stationary or to modified exponential growth phase, the calculated levels of inhibition changed as follows (in %): for wild type mitochondria, from 8 ± 1.12 to 15 ± 2.11 or to 16 ± 2.28 respectively, and for Δ*por1* mitochondria, from 30 ± 2.12 to 18 ± 1.98 or to 17 ± 1.87, respectively.

Levels of pb_2-DHFR bound under "import+MTX" conditions correlate well with levels of inhibition of $O_2^{\bullet-}$ release from mitochondria

Taking into account that the greatest inhibition of $O_2^{\bullet-}$ release was obtained under "import+MTX" conditions, we analyzed the levels of pb_2-DHFR arrested under these conditions. Mge was used as a loading control (Antos et al., 2001a). As shown in Fig.1, an increase in Tom40 levels in wild type mitochondria correlated with increased levels of pb_2-DHFR arrested under "import + MTX" conditions. However, for Δ*por1* mitochondria, in spite of a slight increase in Tom40 levels triggered by the shift from exponential to stationary or modified exponential growth phase, a distinct decrease in levels of pb_2-DHFR arrested under "import + MTX" conditions was observed, although the levels were still higher than for wild type mitochondria. The decrease in levels of arrested pb_2-DHFR may be caused by an increased involvement of the TOM complex in metabolite transport in Δ*por1* mitochondria (Kmita and Budzinska 2000; Antos et al. 2001a). Nevertheless, the observed levels of pb_2-DHFR arrested under "import+MTX" conditions for Δ*por1* and wild type mitochondria isolated from cells in different growth phases correlate well with the observed inhibition of $O_2^{\bullet-}$ release from the mitochondria.

Inhibition of $O_2^{\bullet-}$ release from mitochondria caused by pb_2-DHFR arrest under "import+MTX" conditions requires the integral TOM complex and the presence of the integral outer membrane

As shown in Table 1, the greatest inhibition of $O_2^{\bullet-}$ release was obtained by stable blocking the TOM complex during

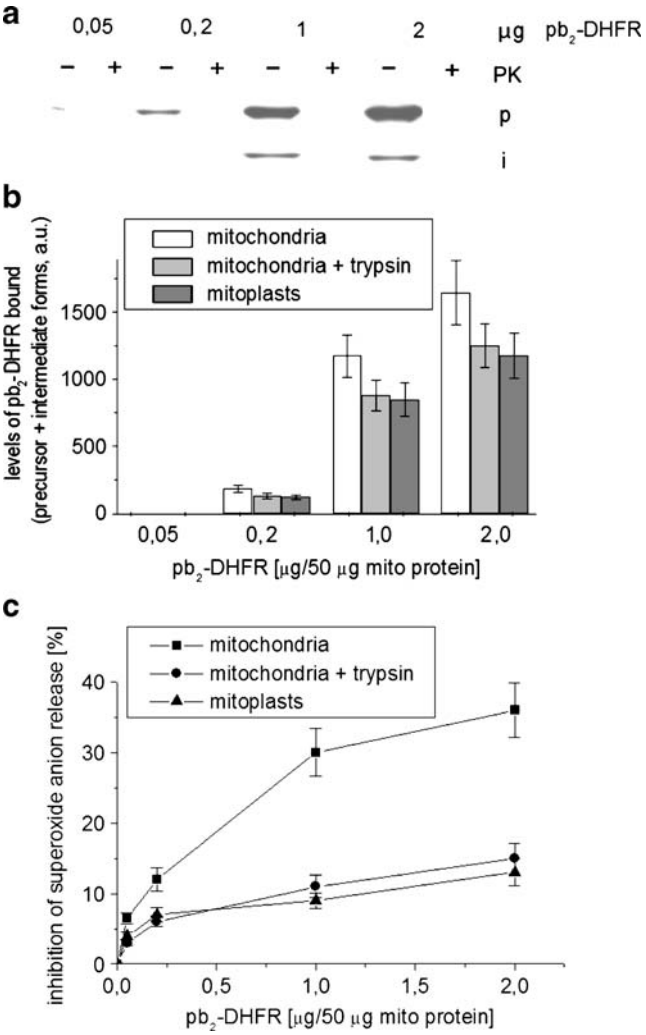

Fig. 2 Effect of increasing concentrations of pb_2-DHFR arrested under "import+MTX" conditions on $O_2^{\bullet-}$ release from Δ*por1* mitochondria isolated from cells in exponential growth phase. The binding of different concentrations of pb_2-DHFR under "import + MTX" conditions was performed as described in "Materials and methods". After incubation mitochondria (native or trypsin-pretreated) or mitoplasts were washed with HS buffer and were used for SDS-PAGE or for measurements of $O_2^{\bullet-}$ release. The levels of arrested pb_2-DHFR were visualized by immunodecoration with anti-DHFR antiserum. (**a**) Example results of a Western blot applied to the analysis and obtained with 50 µg of the studied native mitochondria.+PK denotes proteinase K treatment of mitochondria (250 µg/ml, 10 min at 0ºC); p and i denote precursor and intermediate forms of pb_2-DHFR, respectively. (**b**) Binding levels of increasing concentrations of pb_2-DHFR under "import+MTX" conditions for mitochondria (native or trypsin- pretreated) and mitoplasts. Trypsin pretreatment and mitoplast formation were performed as described in "Materials and methods". (**c**) The effect of trypsin pretreatment and mitoplast formation on inhibition of $O_2^{\bullet-}$ release by pb_2-DHFR arrested under "import + MTX" conditions. Mitochondria and mitoplasts were incubated under "import + MTX" conditions and levels of $O_2^{\bullet-}$ release were calculated as described in "Materials and methods". The data shown in B and C are mean values±SEM of three independent experiments performed for each type of mitochondria and mitoplasts

the resting state ("import+MTX") in Δ*por1* mitochondria isolated from cells in exponential growth phase. These mitochondria simultaneously displayed the highest level of $O_2^{\bullet-}$ release. Therefore, we decided to use the mitochondria to check the effect of blocking the TOM complex with different concentrations of pb_2-DHFR under "import + MTX" conditions. We applied the following concentrations of pb_2-DHFR (in µg per 50 µg of mitochondrial protein): 0.05, 0.2, 1 and 2. As shown in Fig. 2A,B the levels of pb_2-DHFR arrested under "import + MTX" conditions correlated well with the applied concentrations of the fusion protein. Moreover, the increased levels of arrested pb_2-DHFR resulted in increased inhibition of $O_2^{\bullet-}$ release (Fig. 2C). To test whether the inhibition of $O_2^{\bullet-}$ release was indeed caused by blocking the TOM complex, we performed the same measurements for trypsin-pretreated mitochondria. It is well known that trypsin pretreatment of mitochondria results in the removal of receptor subunits of the TOM complex that in turn impairs preprotein binding because the subunits are important for preprotein recognition and translocation (Rapaport et al. 1998; Antos et al. 2001b). Thus, trypsin pretreatment should weaken the TOM complex blockage and subsequently the inhibition of $O_2^{\bullet-}$ release from mitochondria. As shown in Fig. 2B, the trypsin pretreatment indeed decreased levels of pb_2-DHFR arrested under import + MTX" conditions, depending on the applied concentration of the fusion protein. Furthermore, the decrease correlated with inhibitory effects of different amounts of pb_2-DHFR arrested under "import + MTX" conditions on $O_2^{\bullet-}$ release (Fig. 2C). For trypsin-pretreated Δ*por1* mitochondria, the inhibition of $O_2^{\bullet-}$ release by different amounts of pb_2-DHFR decreased as follows (in %): from 6.5±0.8 to 3±0.4 (0.05 µg per 50 µg of mitochondrial protein), from 12 ±1.7 to 6±0.7 (0.2 µg per 50 µg of mitochondrial protein), from 30±3.4 to 11±1.6 (1 µg per 50 µg of mitochondrial protein) and from 36±3.8 to 15±2.2 (2 µg per 50 µg of mitochondrial protein). Therefore, it appears that the observed inhibition of $O_2^{\bullet-}$ release from Δ*por1* mitochondria by pb_2-DHFR arrested under "import+MTX" conditions results from the blockage of the TOM complex. Moreover, Δ*por1* mitoplasts preincubated with the same concentrations of pb_2-DHFR under "import + MTX" conditions bound the protein effectively (Fig. 2B), but the inhibitory effect was distinctly weakened when compared to that calculated for mitochondria (Fig. 2C). The inhibitory effect decreased as follows (in %): from 6.5±0.8 to 4±0.5 (0.05 µg per 50 µg of mitochondrial protein), from 12 ±1.7 to 7±1 (0.2 µg per 50 µg of mitochondrial protein), from 30±3.4 to 9.0±1.1 (1 µg per 50 µg of mitochondrial protein) and from 36±3.8 to 13±1.9 (2 µg per 50 µg of mitochondrial protein). Thus, the observed

decrease in $O_2^{\bullet-}$ release from mitochondria caused by pb_2-DHFR binding under "import+MTX' conditions strongly correlated with the presence of the integral outer membrane.

In summary, the present work has revealed for the first time that the TOM complex contributes to $O_2^{\bullet-}$ release from mitochondria. Using mitochondria of the yeast *Saccharomyces cerevisiae* Δ*por1* mutant depleted of a channel-forming isoform of VDAC, i.e. VDAC1, as well as the isogenic wild type we have shown that the involvement of the TOM complex in $O_2^{\bullet-}$ release from mitochondria is enhanced in the absence of VDAC1 but also occurs in the presence of the isoform, particularly under conditions that trigger high levels of $O_2^{\bullet-}$ release. Thus, as has been suggested by Han et al., (2003), besides VDAC also the TOM complex may form a pathway for $O_2^{\bullet-}$ transport across the mitochondrial outer membrane. Accordingly, Mannella and Kinnally (2008) have proposed that the permeability of the mitochondrial outer membrane may be determined by an ensemble of a relatively small number of predominantly cation-selective channels, including VDAC in different conductance states and the TOM complex.

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