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Involvement of the TOM complex in external NADH transport into yeast mitochondria depleted of mitochondrial porin1

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This paper is dedicated to Professor Jan Michejda (1927–1999).

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Abstract

The protein(s) responsible for metabolite transport through the outer membrane of the yeast *Saccharomyces cerevisiae* mitochondria depleted of mitochondrial porin (also known as voltage-dependent anion selective channel), termed here porin1, is (are) still unidentified. It is postulated that the transport may be supported by the protein import machinery of the outer membrane, the TOM complex (translocase of the outer membrane). We demonstrate here that in the absence of functional porin1, the blockage of the TOM complex by the fusion protein termed pb_2-DHFR (consisting of the first 167 amino acids of yeast cytochrome b_2 preprotein connected to mouse dihydrofolate reductase) limits the access of external NADH to mitochondria. It was measured by the ability of the blockage to inhibit external NADH oxidation by the proper dehydrogenase located at the outer surface of the inner membrane. The inhibition depends on external NADH concentration and increases with decreasing amounts of the substrate. In the presence of 1 μg of pb_2-DHFR per 50 μg of mitochondrial protein almost quantitative inhibition was observed when external NADH was applied at the concentration of 70 nmol per mg of mitochondrial protein. On the other hand, external NADH decreases the levels of pb_2-DHFR binding at the *trans* site of the TOM complex in porin1-depleted mitochondria in a concentration-dependent fashion. Our data define an important role of the TOM complex in the transport of external NADH across the outer membrane of porin1-depleted mitochondria.

Keywords: Metabolite transport; Mitochondrial porin or porin1 (voltage-dependent anion selective channel); Porin1-depleted mitochondrion; Translocase of the outer membrane (TOM) complex

1. Introduction

The effective transport of metabolites through both mitochondrial membranes is crucial to the energetic and metabolic functions of mitochondria. While the inner membrane contains specific metabolite carriers, the permeability of the outer one is thought to be based in diffusion pores formed by mitochondrial porin, also known as VDAC (voltage-dependent anion-selective channel) [1–4]. However, the results of studies on mutants of the yeast *Saccharomyces cerevisiae* genetically depleted of the *POR1* gene encoding mitochondrial porin [5–7], termed also porin1, lead to the conclusion that other protein(s) may contribute to metabolite transport across the mitochondrial outer membrane. Yeast cells deleted for *POR1* gene (Δ*por1*) are viable on non-fermentable carbon sources which indicates

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that in the absence of porin1, the mitochondria are able to exchange metabolites across the outer membrane. The protein(s) that could functionally substitute for porin1 is (are) also present in wild type mitochondria as there are no differences in the electrophoretic protein pattern of the outer membrane between Δ*por1* and wild type mitochondria [8].

Recently, a new gene (*POR2*) was identified in the *S. cerevisiae* encoding a protein that, when overexpressed, suppressed the deletion of *POR1* gene [5]. However, most of the residual permeability observed for external NADH in porin1-depleted mitochondria is also found in mitochondria isolated from Δ*por1*-Δ*por2* cells which indicates that other outer membrane protein may participate in the passage of metabolites through the membrane [9]. The protein might be identical with PSC (peptide-sensitive channel), present in *S. cerevisiae* mitochondria independently of porin1 [10], since the electrophysiological characteristics of PSC correspond to results obtained for mitochondria of different porin1-depleted mutants of *S. cerevisiae* with both electrophysiological [8] or functional [9,11] approaches. The conductivity of PSC is modulated by basic peptides, including peptides which serve as mitochondrial targeting sequences. PSC was demonstrated to participate in the translocation of peptides and proteins through the outer membrane [12–13]. Moreover, PSC is regarded as being identical with Tom 40 [14–16], the putative translocation pore of the outer membrane protein import machinery (translocase of the outer membrane, the TOM complex [17]). Thus, if the TOM complex indeed participates in the transport of metabolites in porin1-depleted mitochondria, interference between protein and metabolite transport through the outer membrane should occur, even in the presence of the protein encoded by the *POR2* gene.

In *S. cerevisiae* mitochondria the access of external NADH to the respiratory chain is restricted only by the outer membrane as electrons from the substrate are accepted by the rotenone-insensitive and antimycin A-sensitive NADH dehydrogenase located on the outer surface of the inner membrane [18]. Therefore, the oxidation of external NADH by mitochondria provides a simple approach for the estimation of metabolite passage through the outer membrane [9,11]. Here we demonstrate that in the absence of porin1, the access of external NADH to the intermembrane space is restricted by chemical amounts of the fusion protein pb_2-DHFR (consisting of the first 167 amino acids of yeast cytochrome b_2 preprotein connected to mouse dihydrofolate reductase) accumulated at the *trans* site of the TOM complex. We also present that external NADH itself may limit the extent of the TOM complex blockage by the applied fusion protein.

2. Materials and methods

2.1. Isolation of mitochondria and mitoplasts

A wild type strain of *S. cerevisiae* DBY747 (*Matα*, *ura3–52*, *leu2–3*, *leu2–112*, *his3–Δ1*, *trp1–289*) and porin1-depleted mutant B5, derived from the above parent strain [6,19] were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5. Growth was monitored by determining the optical density (OD) at 546 nm and the cells were collected in log phase, at an OD of about 1. Mitochondria were isolated according to published procedure [20]. Mitoplasts were obtained by the swelling–shrinking procedure [20]. The swelling buffer contained 20 mM HEPES pH 6.9 and 0.2% bovine serum albumin (BSA). The shrinking buffer contained 1.8 M mannitol, 4 mM $MgCl_2$ and 0.2% BSA. The integrity of the outer mitochondrial membrane was monitored by the permeability of the membrane for exogenous cytochrome *c* [21] or by immunodecoration with antisera against marker proteins of the intermembrane space and mitochondrial matrix. (The antisera were a kind gift of Professor W. Neupert.)

2.2. Accumulation of pb_2-DHFR within the outer mitochondrial membrane

The high level expression and purification of a fusion protein called pb_2-DHFR was performed as described in [22]. The fusion protein consists of the first 167 amino acid residues of yeast cytochrome b_2 precursor comprising the mitochondrial targeting sequence with the intermembrane space sorting signal and the entire mouse dihydrofolate reductase. Radiolabeled pb_2-DHFR was synthesized in rabbit reticu-

locyte lysate (Promega) in the presence of [^{35}S]-methionine after in vitro transcription by SP6 polymerase from transcription vector pGEM4 containing the gene of pb_2-DHFR. (The *Escherichia coli* strain expressing pb_2-DHFR and the plasmid containing the fusion protein gene were kind gifts of Professor W. Neupert.)

Mitochondria 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, NADH at various concentrations, 0.16 μg of valinomycin and 6 μg oligomycin per mg of mitochondrial protein) for 15 min at 25°C in the presence of 1 μg pb_2-DHFR per 50 μg of mitochondrial protein [23] or proper amounts of its radiolabeled form. For an import reaction valinomycin and oligomycin were omitted in the import buffer. To assay for the fusion protein binding at the *trans* site of the TOM complex, mitochondria were washed with HS (high salt) buffer (250 mM sucrose, 10 mM MOPS–KOH pH 7.2, 120 mM KCl) [24–25]. When indicated, mitochondria or mitoplasts were treated with proteinase K (250 μg/ml, 10 min at 0°C) and washed with SM buffer (250 mM sucrose, 10 mM MOPS–KOH pH 7.2) in the presence of 1 mM phenylmethylsulfonyl fluoride. In experiments using trypsin pretreatment, mitochondria were incubated with the protease (50 μg/ml) for 15 min on ice and then soybean trypsin inhibitor was added (1 mg/ml) prior to the accumulation reaction. In control samples (−trypsin) the protease and its inhibitor was applied simultaneously. After reisolation (10 min, 12 000×*g*), the organelles were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [26]. Accumulation of pb_2-DHFR was visualized by the enhanced chemiluminescence (ECL) methods followed by immunodecoration with anti-mouse DHFR antiserum or fluorography and quantified by ScanPack III. (The antiserum was a kind gift of Professor W. Neupert.)

2.3. Determination of the rate of NADH oxidation

Mitochondria or mitoplasts were incubated as for the fusion protein accumulation reaction (see Section 2.2) in the presence of different external NADH concentrations and NADH oxidation was monitored spectrophotometrically (UV 1602 Shimadzu).

2.4. Other methods

Protein concentrations were measured by the method of Bradford. The respiration of mitochondria and mitoplasts was monitored at 25°C with a Rank oxygen electrode in the incubation volume of 0.5 ml. Changes of the inner membrane potential ($\Delta\psi$) were monitored with a tetraphenylophosphonium-specific electrode as described in [27].

3. Results

3.1. Efficient blockage of the TOM complex in wild type and porin1-depleted mitochondria

To establish conditions under which the TOM complex would be blocked efficiently we used chemical amounts of a fusion protein called pb_2-DHFR. We studied its accumulation at the outer membrane of isolated wild type and porin1-depleted mitochondria. According to published data, dissipation of the inner membrane potential ($\Delta\psi$) completely blocks preprotein transfer to the inner membrane. Instead, the preprotein is bound to the TOM complex at its *trans* site facing the intermembrane space [24–25,28]. We performed a binding reaction of pb_2-DHFR to uncoupled mitochondria (MITO) and to uncoupled mitoplasts (MTP, mitochondria after the removal of the outer membrane). The latter were used as a control of an insertion place of the fusion protein in the absence of $\Delta\psi$. Since comparable results were observed with wild type and porin1-depleted mitochondria, in Fig. 1 we present only those obtained for mutant mitochondria. After the binding reaction mitochondria were washed under high salt conditions to eliminate pb_2-DHFR binding to the cytosolic surface of the TOM complex (the *cis* site) [23–24] and translocation of the fusion protein through the outer membrane was estimated by treatment with proteinase K. In the absence of the inner membrane potential ($-\Delta\psi$)(Fig. 1A), pb_2-DHFR was mainly bound at the level of the outer membrane but not at the level of the inner one as uncoupled mitoplasts bound only a trace amount of pb_2-DHFR. The fusion protein bound at the level of the outer membrane still remained accessible to the externally added protease and was not processed by matrix processing pepti-

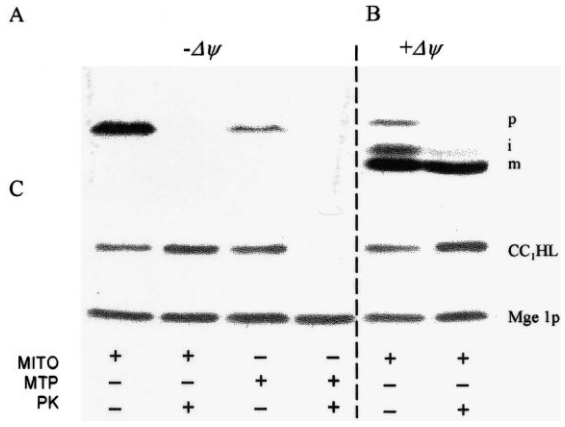

Fig. 1. Accumulation of pb_2-DHFR within the outer membrane of porin1-depleted mitochondria. (A) Binding of pb_2-DHFR with mutant mitochondria (MITO) and mitoplasts (MTP) in the absence of the inner membrane potential ($-\Delta\psi$). (B) Import of pb_2-DHFR into porin1-depleted mitochondria (MITO) in the presence of the inner membrane potential ($+\Delta\psi$). (C) Quantity and quality control of results shown in A and B. In all experiments external NADH at a concentration of 4 μmol per mg of mitochondrial protein was applied as the respiratory substrate. After washing with HS buffer followed by the accumulation or import reaction, all samples were divided in two and proteinase K was added to one half (+PK) and omitted in the second one (−PK). They were next washed with SM buffer and reisolated samples were analyzed by SDS–PAGE and immunoblotted with anti-DHFR (A, B) as well as anti-CC_1HL and anti-Mge1p (C) antisera.

dase (MPP) as only the precursor form of the protein (p) was detected. Further, the binding reaction was performed for radiolabeled pb_2-DHFR using mitochondria preincubated under the *trans* site binding conditions in the presence or absence of chemical amounts of the fusion protein (1 μg per 50 μg of mitochondria). After preaccumulation of pb_2-DHFR at the *trans* site of the TOM complex mitochondria bound distinctly lower amounts of its radiolabeled form than in the absence of the previous accumulation of the fusion protein (not shown). These data suggest that under applied conditions pb_2-DHFR was only partially translocated through the outer membrane and blocked the TOM complex efficiently. To check import competence of the applied fusion protein, i.e. to test whether its insertion into the inner membrane is specifically inhibited by the absence of $\Delta\psi$, an import reaction was performed using coupled mitochondria ($+\Delta\psi$) (Fig. 1B). A considerable fraction of pb_2-DHFR was processed into external protease-sensitive intermediate (i) and external protease-resistant mature (m) forms indicating that in the presence of $\Delta\psi$, the fusion protein was efficiently imported into mitochondria. Fig. 1C serves as a control of results shown in Fig. 1A,B. Only selective rupture of the outer membrane (MTP) allowed proteinase K to digest proteins localized in the intermembrane space (e.g. CC_1HL) while those located in the matrix (e.g. Mge1p) were present in similar amounts. Thus, a partial translocation of chemical amounts of pb_2-DHFR within the outer membrane due to the collapse of the inner membrane potential leads to accumulation of the fusion protein resulting in effective blockage of the TOM complex.

3.2. *pb_2-DHFR accumulated within the outer membrane inhibits the access of external NADH into porin1-depleted uncoupled mitochondria*

We next investigated whether the accumulation of pb_2-DHFR during its translocation through the outer membrane could restrict the access of external NADH into the intermembrane space of mitochondria depleted of porin1. For that purpose we examined the influence of the presence of chemical amounts of pb_2-DHFR bound to the *trans* site of the TOM complex on the rate of external NADH oxidation (Fig. 2). When external NADH was applied at a concentration of 1.4 μmol per mg of mitochondrial protein, binding of 1 μg pb_2-DHFR per 50 μg of mitochondria under the *trans* site conditions resulted in inhibition of the rate of its oxidation by about 25%. The inhibition was significantly weakened (approximately 60%) by the trypsin pretreatment (causing the removal of receptors important for preprotein recognition and their subsequent translocation which results in their decreased levels of binding at the *trans* site of the TOM complex). The removal of the outer membrane of mutant mitochondria eliminated the effect of pb_2-DHFR on external NADH oxidation. The effect was also absent from wild type mitochondria. It should be emphasized here that in the absence of mitochondria, pb_2-DHFR 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). Therefore the results suggest that the fusion protein accumulated within the outer membrane may hamper external NADH

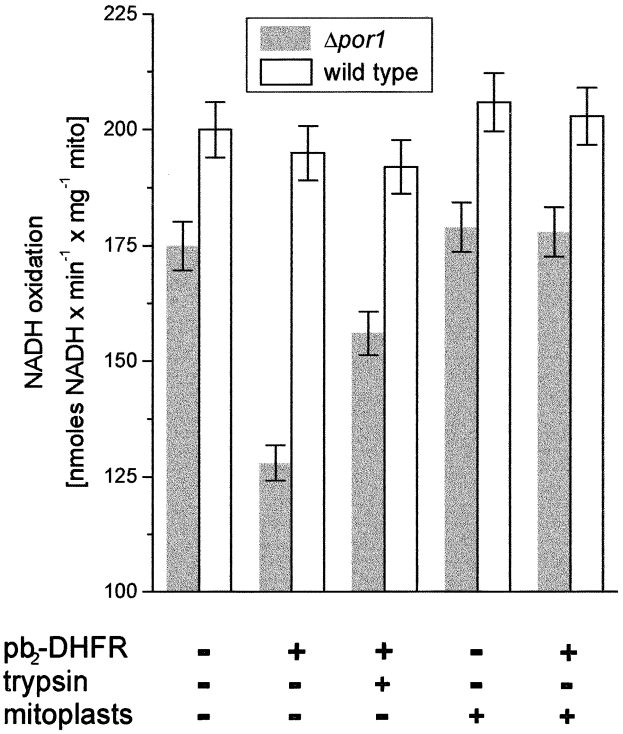

Fig. 2. Oxidation of external NADH by porin1-depleted uncoupled mitochondria in the presence of pb_2-DHFR bound at the *trans* site of the TOM complex. Mitochondria or mitoplasts were incubated as for the accumulation reaction and the rate of external NADH was determined spectrophotometrically. From left to right the first three double bars represent the rates of NADH oxidation calculated for mitochondria, the next two for mitoplasts. When indicated mitochondria were trypsin pretreated. NADH was applied at concentration of 1.4 mol per mg mitochondrial protein. Data are mean values ±S.E.M. of three independent experiments.

passage across the membrane of porin1-depleted mitochondria.

We next tested whether the inhibitory effect of pb_2-DHFR on the rate of external NADH oxidation could depend on applied concentrations of NADH. We assumed that a lowering of external NADH concentrations might weaken its access to the TOM complex, leading to a stronger inhibition of its oxidation by pb_2-DHFR while higher concentrations of the substrate ensuring its better access to the transport pathway would result in a weaker effect of pb_2-DHFR. As shown in Table 1, in the presence of the same amount of pb_2-DHFR (1 μg per 50 μg of mitochondria), decreasing concentrations of external NADH resulted in higher levels of the inhibition of its oxidation. Almost quantitative inhibition was achieved at approximately 0.07 μmol NADH per 1 mg of mitochondrial protein. Thus, the translocation of pb_2-DHFR through the outer membrane of porin1-depleted mitochondria appears to restrict the simultaneous transport of external NADH in an NADH concentration-dependent fashion.

3.3. *External NADH decreases the levels of pb_2-DHFR binding to the trans site of the TOM complex in porin1-depleted uncoupled mitochondria*

In order to investigate whether the transport of external NADH through the outer membrane of porin1-depleted mitochondria (being affected by pb_2-DHFR, see Section 3.2) could mutually restrict the translocation of pb_2-DHFR through the membrane, we performed the *trans* site binding reaction in the absence of external NADH and in the presence of increasing concentrations of the substrate. We assumed that the action of the respiratory chain was not important here because the binding of pb_2-DHFR to the *trans* site of the TOM complex proceeded in the absence of the inner membrane potential. It should be emphasized here that in the presence of chosen external NADH concentrations the effect of pb_2-DHFR changed from very strong (0.2 μmol NADH per mg of mitochondrial protein) to rather weak (4 μmol NADH per mg of mitochondrial protein) (see Table 1). As shown in Fig. 3, in

Table 1
The effect of external NADH concentration on the levels of its oxidation inhibition by pb_2-DHFR bound at the *trans* site of the TOM complex of porin1-depleted mitochondria

NADH concentration (μmol × mg^{-1} mitochondrial protein)	Inhibition of NADH oxidation (%)
4	15
1.4	25
0.7	40
0.2	90
0.07	100

The rate of external NADH oxidation was determined spectrophotometrically in the presence of 1 μg of pb_2-DHFR per 50 μg of mitochondrial protein. For applied external NADH concentrations control measurements were made in the absence of pb_2-DHFR and used to calculate the levels of inhibition. Presented data are the result of a typical experiment.

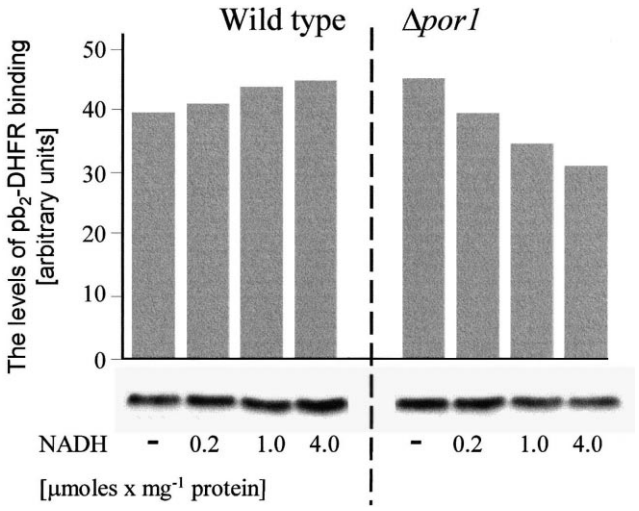

Fig. 3. Binding of pb_2-DHFR to the *trans* site of the TOM complex of porin1-depleted uncoupled mitochondria in the absence and presence of external NADH. During the accumulation reaction external NADH was omitted or applied at concentrations of 0.2, 1 and 4 μmol per mg of mitochondrial protein. After washing with HS buffer samples were separated by SDS–PAGE. Accumulation of pb_2-DHFR was visualized by immunodecoration with anti-mouse-DHFR antiserum and quantified by ScanPack III. Binding of pb_2-DHFR varied by not more than 12% in various experiments.

the case of wild type mitochondria, the amount of the fusion protein bound in a salt-resistant way (at the *trans* site of the TOM complex) increased slightly in the presence of increasing concentrations of external NADH and the lowest levels of binding was observed in the absence of the substrate. However, with porin1-depleted mitochondria the highest amount of pb_2-DHFR was bound to the *trans* site of the TOM complex in the absence of external NADH and increasing concentrations of the substrate lowered the levels of the fusion protein binding. Further, the integrity of the outer mitochondrial membrane was estimated for all studied samples by immunodecoration with antisera against marker proteins of the intermembrane space and mitochondrial matrix (not shown, see Fig. 1C). This allowed the conclusion that the differences in the levels of pb_2-DHFR binding to the *trans* site of the TOM complex did not result from the damage of the outer membrane. We infer from these data that in the absence of porin1, external NADH may restrict the access of pb_2-DHFR to the *trans* site of the TOM complex in a concentration-dependent fashion. The hypothesis seems to be supported by the observation that in porin1-depleted mitochondria, external NADH restricts the access of presequence peptides of mitochondrial proteins to the inner membrane (O. Stobienia, unpublished results).

4. Discussion

Here we report data important for the identification of the protein(s) taking over the function of mitochondrial porin (also known as VDAC and termed here porin1) in mitochondria of *S. cerevisiae* depleted of the protein. It is clear now that porin1 is not the only protein responsible for the transport of metabolites through the outer mitochondrial membrane, as several porin1-depleted yeast mutants have been found to be viable on non-fermentable carbon source [5–7,19]. It was suggested previously [9,11,29] that in porin1-depleted mitochondria the function of porin1 can be taken over by another outer membrane channel such as the PSC, described among others in mutant B5, being the object of present studies [10]. The PSC is now regarded as being identical with Tom 40 [13–14,16], the putative translocation pore of the outer membrane protein import machinery, i.e. the TOM complex [17].

We have demonstrated here that the absence of porin1 leads to interference between the fusion protein pb_2-DHFR and external NADH transport through the outer membrane. Firstly, the access of external NADH into the intermembrane space is limited by the simultaneous blockage of the TOM complex by chemical amounts of pb_2-DHFR bound at the *trans* site as measured by adequate changes in the rate of external NADH oxidation. The effect of the fusion protein on external NADH accessibility appears to be specifically connected with the TOM complex as its becomes weaker after the removal of surface receptors of the complex and is diminished completely after the removal of the outer membrane. Secondly, the interference between pb_2-DHFR and external NADH seems to be concentration-dependent, indicating the competition between both species for transport through the outer membrane. In the presence of an unchanged amount of pb_2-DHFR, the lowering of applied external NADH concentrations results in a stronger inhibition of its oxidation

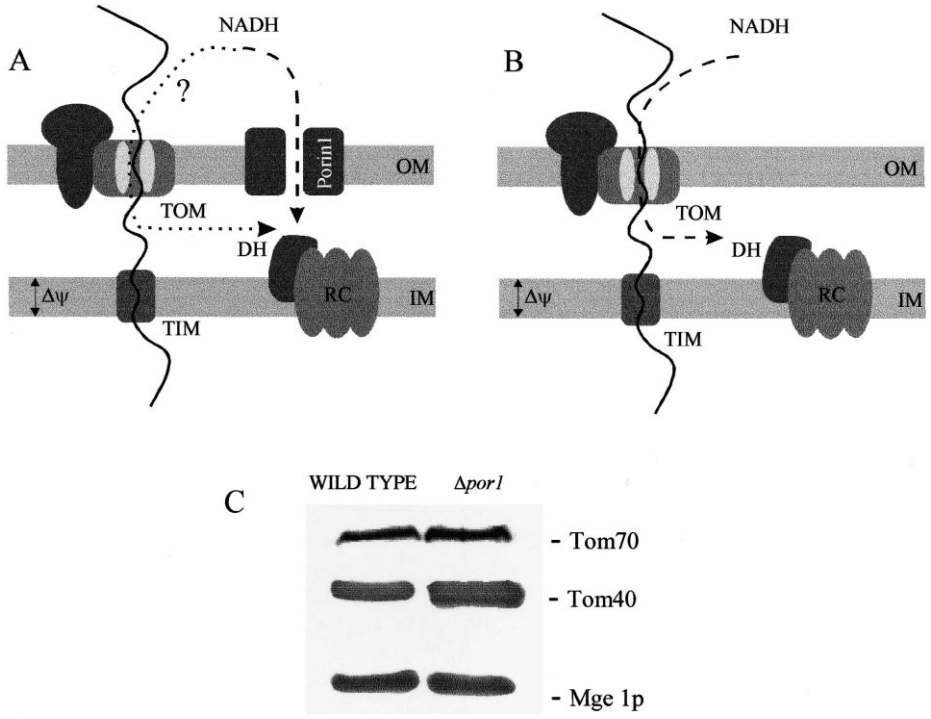

Fig. 4. Working model for the TOM complex as the possible pore for metabolite transport across the outer membrane of porin1-depleted mitochondria. (A) In wild type mitochondria, porin1 and the TOM complex seem to form independent pathways of molecule transport through the membrane. (B) In the absence of porin1, its function may be taken over by the TOM complex. (C) The levels of Tom40 and Tom70 expression in wild type and porin1-depleted mitochondria. Samples were separated by SDS–PAGE. The levels of Tom40, Tom70 and Mge1p were visualized by immunodecoration with proper antisera. OM, the outer membrane; DH, the external NADH dehydrogenase; RC, respiratory chain; IM, the inner membrane.

up to the maximal level of 100%. On the other hand, the pb_2-DHFR binding at the *trans* site of the TOM complex is decreased in the presence of external NADH, especially when higher concentrations of the substrate are applied. It should be noted here that these results were obtained only with porin1-depleted mitochondria whereas with wild type mitochondria no interference between external NADH and pb_2-DHFR under applied conditions was observed.

The presented data suggest that external NADH and pb_2-DHFR may use the same pathway to cross the mitochondrial outer membrane when porin1 is absent. As shown in Fig. 4, external NADH is transported through the outer membrane as it is easily oxidized at the level of the inner membrane both in wild type and porin1-depleted mitochondria [9,11]. In wild type mitochondria the transport of external NADH is mainly supported by porin1 [9]. In the case of porin1-depleted mitochondria, external NADH crosses the outer membrane due to still unidentified protein(s). In wild type mitochondria (Fig. 4A) the transport of metabolites and preproteins seems to proceed independently as any direct interference between both processes was not observed for wild type mitochondria under applied *trans* site conditions. Thus, one can conclude that the porin1-supported transport system probably acts independently of the mitochondrial import machinery (see also [12]). However, in the absence of functional porin1 (Fig. 4B), transport of external NADH may be preserved by a protein being a component of the TOM complex. The TOM complex is known to contain a cation-selective high-conductance channel. A central role in organizing the channel is assigned to Tom40 [13,16,30]. Studied porin1-depleted mitochondria contain higher levels of Tom40 when compared to wild type mitochondria (Fig. 4C). The upregulation is also observed for other Tom proteins, e.g. for Tom70 belonging to import receptors, which might enable the TOM complex to participate in metabolite transport. The pore for metabolites formed by the

TOM complex might be responsible for the residual permeability of the outer membrane for external NADH observed for mitochondria of the *S. cerevisiae* mutant depleted of both *POR1* and *POR2* genes [9].

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