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Under Conditions of Insufficient Permeability of VDAC1, External NADH May Use the TOM Complex Channel to Cross the Outer Membrane of *Saccharomyces cerevisiae* Mitochondria

Nina Antos,[1] Olgierd Stobienia,[1] Małgorzata Budzińska,[1] and Hanna Kmita[1,2]

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Thus far, only three channel-forming activities have been identified in the outer membrane of the yeast *Saccharomyces cerevisiae* mitochondria. Two of them, namely the TOM complex channel (translocase of the outer membrane) and the PSC (peptide-sensitive channel) participate in protein translocation and are probably identical, whereas a channel-forming protein called VDAC (voltage-dependent anion channel) serves as the major pathway for metabolites. The VDAC is present in two isoforms (VDAC1 and VDAC2) of which only VDAC1 has been shown to display channel-forming activity. Moreover, the permeability of VDAC1 has been reported to be limited in uncoupled mitochondria of *S. cerevisiae*. The presented data indicate that in *S. cerevisiae*-uncoupled mitochondria, external NADH, applied at higher concentrations (above 50 nmoles per 0.1 mg of mitochondrial protein), may use the TOM complex channel, besides VDAC1, to cross the outer membrane. Thus, the permeability of VDAC1 could be a limiting step in transport of external NADH across the outer membrane and might be supplemented by the TOM complex channel.

KEY WORDS: *Saccharomyces cerevisiae* mitochondria; external NADH transport; isoform1 of voltage-dependent anion channel (VDAC1); TOM complex channel.

INTRODUCTION

The major pathway for metabolite transport across the outer mitochondrial membrane is formed by a channel called VDAC (voltage-dependent anion-selective channel) also known as mitochondrial porin (Schein *et al.*, 1976; Colombini, 1979; Benz, 1994; Colombini *et al.*, 1996). It has been demonstrated that in one organism, different isoforms of VDAC may be present, displaying different properties and probably playing different roles (Blachly-Dyson *et al.*, 1993, 1997; Elkeles *et al.*, 1997; Sampson *et al.*, 1997; Xu *et al.*, 1999). Usually, in a given organism, a channel-forming activity has been found for more than one of VDAC isoforms, e.g., in humans (Blachly-Dyson *et al.*, 1993), in wheat (Elkeles *et al.*, 1997), or in mice (Xu *et al.*, 1999). However, in the yeast *Saccharomyces cerevisiae* only one of two the VDAC isoforms has been proved to form a channel (Blachly-Dyson *et al.*, 1997) and to be crucial to external NADH transport across the outer membrane (Lee *et al.*, 1998). This VDAC isoform is called VDAC1 (or porin1) and its properties are highly conserved among other species. The second yeast VDAC protein, VDAC2, probably has lost the channel-forming activity and its role in the outer membrane permeability to external NADH is rather minimal (Lee *et al.*, 1998).

It is now clear that VDAC1 is not the only protein supporting metabolite transport across the outer membrane of *S. cerevisiae* mitochondria. Yeast cells genetically depleted of the *POR1* gene encoding VDAC1 (Guo

[1] Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Poznan University, 61-701 Poznan, Poland.
[2] To whom correspondence should be addressed at email: kmita@main.amu.edu.pl

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and Lauquin, 1986; Dihanich *et al.*, 1987; Michejda *et al.*, 1990; Blachly-Dyson *et al.*, 1997) are viable on nonfermentable carbon sources. Thus, in the absence of VDAC1, the mitochondria are able to exchange metabolites across the outer membrane. Most of the residual permeability observed for external NADH in VDAC1-depleted *S. cerevisiae* mitochondria is also found in mitochondria devoid of both VDAC isoforms, which indicates that other outer membrane protein(s) may participate in the passage of metabolites through the membrane (Lee *et al.*, 1998). The protein(s) that could functionally substitute for VDAC1 is (are) also present in wild-type mitochondria, as there are no differences in the electrophoretic protein pattern of the outer membrane between Δ*por*1 and wild-type mitochondria (Dihanich *et al.*, 1989; our unpublished results).

Thus far, only two channels distinct from VDAC1 have been identified in the outer membrane of *S. cerevisiae* mitochondria. These are the peptide-sensitive channel (PSC) (Thieffry *et al.*, 1988; Fevre *et al.*, 1990) and the TOM complex channel (Hill *et al.*, 1998; Kuenkele *et al.*, 1998a,b). The TOM complex, also known as the translocase of the outer membrane, participates in protein import into mitochondria (Lill and Neupert, 1996). It has been proved that both the TOM complex channel and the PSC serve as the preprotein-conducting channel of the outer mitochondrial membrane (Vallette *et al.*, 1994; Hill *et al.*, 1998; Kuenkele *et al.*, 1998a,b). Moreover, the ion-conducting properties of the purified TOM complex resemble those of the PSC. Therefore the both channels are thought to be identical (Juin *et al.*, 1997; Kuenkele *et al.*, 1998a,b).

It has been reported that the TOM complex channel may participate in transport of molecules other than proteins, e.g., tRNAs (Tarassov *et al.*, 1995). It is also postulated that in mitochondria of VDAC1-depleted mutant of *S. cerevisiae*, the TOM complex channel is involved in the permeability of the outer membrane for external NADH (Kmita and Budzińska, 2000). In *S. cerevisiae* mitochondria, external NADH is oxidized by the rotenone-insensitive and antimycin A-sensitive NADH dehydrogenase located on the outer surface of the inner membrane (De Vries and Marres, 1987). Therefore, oxidation of external NADH provides a simple approach for the estimation of metabolite passage through the outer membrane (Michejda *et al.*, 1994; Lee *et al.*, 1998) as its access to the respiratory chain is restricted only by the outer membrane. Here, we report that even in the presence of functional VDAC1, the TOM complex may contribute to external NADH transport across the outer membrane of *S. cerevisiae* mitochondria.

MATERIALS AND METHODS

Isolation of Mitochondria and Mitoplasts

A wild-type strain of *Saccharomyces cerevisiae* M3 (*MATα*, *lys2*, *his4*, *trp1*, *ade2*, *leu2*, *ura3*) (Blachly-Dyson *et al.*, 1997) were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol), at pH 5.5. Their growth was monitored by determining the optical density (OD) at 546 nm and the cells were collected in the log phase, at OD of about 1. Mitochondria were isolated according to the procedure published by Daum *et al.* (1982). Mitoplasts were obtained by the swelling-shrinking procedure (Daum *et al.*, 1982). 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 to exogenous cytochrome *c* (Douce *et al.*, 1984) or by immunodecoration with antisera against marker proteins of the intermembrane space (cytochrome c_1 heme lyase) and mitochondrial matrix (Mge1) (not shown).

Synthesis of pb_2-DHFR

The fusion protein called pb_2-DHFR 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 (pb_2) and the entire mouse dihydrofolate reductase (DHFR). The high level expression and purification of pb_2-DHFR was performed as described by Wienhues *et al.* (1992). A radiolabeled form of pb_2-DHFR was synthesized in rabbit reticulocyte 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.

Binding and Import of pb_2-DHFR

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 3 μg of pb_2-DHFR per 50 μg of mitochondrial protein (Dekker *et al.*, 1997) or proper amounts of its radiolabeled form. For an import reaction, valinomycin and oligomycin were omitted in the

import buffer. Accumulation of pb_2-DHFR within both mitochondrial membranes of coupled mitochondria (resting state + MTX) was performed as described by Voos *et al.* (1994) in the import buffer and in the presence of 2 μM cross-linking agent methotrexate (MTX). To assay for pb_2-DHFR binding inside mitochondria, samples were washed with HS (high-salt) buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2, 120 mM KCl) (Rapaport *et al.*, 1997, 1998). When indicated, after the washing mitochondria or mitoplasts were resuspended in SM buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2) and treated with proteinase K (250 μg/ml, 10 min at 0°C), the reaction 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 binding reaction. In control samples (-trypsin), the protease and its inhibitor were applied simultaneously. After reisolation (10 min, $12{,}000 \times g$), the organelles were subjected to SDS–PAGE (Lämmli, 1970). Binding of pb_2-DHFR was visualized by fluorography or the ECL methods (Amersham) following immunodecoration with anti-mouse DHFR antiserum (standard procedure) and quantified by ScanPack 3.0.

Determination of the Rate of NADH Oxidation

Mitochondria (native or trypsin pretreated) or mitoplasts were incubated as for the pb_2-DHFR binding reaction (see above) in the presence of different external NADH concentrations and NADH oxidation was monitored spectrophotometrically (UV 1602 Shimadzu).

Conductance Measurements in Planar Phospholipid Membranes

Isolation of VDAC1 was performed as described by De Pinto *et al.* (1987). Native or trypsin pretreated mitochondria (see above) were incubated for 30 min at 0°C in the solubilization buffer containing 3% Triton X-100, 10 mM Tris-HCl (pH 7.0), and 1 mM EGTA at the final concentration of 5 mg of mitochondrial protein per ml. The solubilization mixture was centrifuged for 30 min at $15{,}000 \times g$ and 0.6-ml aliquot of the supernatant was loaded onto a dry hydroxyapatite/celite column (0.6 g, at ratio 2:1). The elution was performed with the solubilization buffer. The first 0.6-ml sample of the eluate was checked by SDS–PAGE and used for reconstitution. The planar phospholipid membrane experiments were performed according to Benz *et al.* (1978). Membranes were formed from 2% (w/v) solution of diphytanoyl phosphatidylcholine (DPPC, Avanti Polar Lipids, Alabaster, AL) or 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 which were filled with unbuffered 1 M KCl, pH 7.0 (not shown).

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 tetraphenylphosphonium (TPP^+) -specific electrode as described by Kamo *et al.* (1979).

RESULTS AND DISCUSSION

According to published data (Mayer *et al.*, 1995; Rapaport *et al.*, 1997, 1998), in the absence of the inner membrane potential ($\Delta\psi$), import of preproteins into mitochondria is arrested at the level of the outer membrane, after presequence translocation and binding to the TOM complex at the *trans* site facing the intermembrane space. Since the translocation is supported by the protein import channel within the TOM complex (Hill *et al.*, 1998; Kuenkele *et al.*, 1998a,b), the resulting translocation intermediate bound at the *trans* site of the TOM complex should stick in the channel causing its blockage (Kmita and Budzińska, 2000). In the presence of $\Delta\psi$, preproteins cross both mitochondrial membranes because of the TOM complex and its counterpart in the inner membrane, namely, the TIM complex. The import process requires close opposition of the complexes enabling the formation of the translocation contact sites. Within these sites, translocated preproteins spanning both mitochondrial membranes link TOM and TIM complexes (Lill and Neupert, 1996). It is possible to arrest a translocated preprotein within the both complexes by stabilization of the folded state of its part remaining outside mitochondria. For example methotrexate (MTX) is well known to stabilize the folding state of fusion proteins containing dihydrofolate reductase (DHFR), preventing this part of fusion proteins from translocation (Voos *et al.*, 1994; Dekker *et al.*, 1997). Taking these data into account, we blocked the TOM complex channel in yeast mitochondria under the following conditions: (1) the *trans* site binding conditions (uncoupled mitochondria) and (2) the resting state + MTX conditions (coupled mitochondria in the

presence of MTX). For that purpose, we used chemical amounts of pb_2-DHFR (3 μg per 50 μg of mitochondrial protein) being a fusion protein between the N-terminal 167 amino acids of yeast cytochrome b_2 preprotein and mouse dihydrofolate reductase. As shown in Fig. 1A, in the presence of $\Delta\psi$, incubation of pb_2-DHFR (p) with isolated yeast mitochondria (MITO) resulted in formation of the intermediate form (i) and the mature form (m) of the preprotein, sensitive and resistant to externally added proteinase K (PK), respectively. Thus, in the presence of $\Delta\psi$, pb_2-DHFR was efficiently imported into yeast mitochondria. In the presence of MTX (MITO + MTX), correctly translocated pb_2-DHFR accumulated as the precursor form (p) partially processed by matrix protease into the intermediate form, both of which being accessible to externally added proteinase K. The accessibility to the proteases displayed by the intermediate form allow the conclusion that it became arrested simultaneously within the TOM and TIM complexes. Dissipation of $\Delta\psi$ eliminated processing of pb_2-DHFR into its intermediate and mature forms, but the precursor form (p) was still bound by yeast mitochondria. Uncoupled mitoplasts (MTP), obtained due to removal of the outer mitochondrial membrane, bound only trace amounts of the fusion protein. Further, pb_2-DHFR bound by uncoupled mitochondria was digested by externally added proteinase K. These data indicate that the binding of pb_2-DHFR with uncoupled yeast mitochondria was confined to the outer membrane. After the binding reaction, but prior to proteinase K treatment, uncoupled mitochondria or mitochondria incubated under the resting state + MTX conditions were washed under high salt conditions (120 mM KCl) to remove pb_2-DHFR bound at the cytosolic surface of the TOM complex (Rapaport *et al.*, 1997, 1998). Therefore, in Fig. 1A, bands corresponding to pb_2-DHFR bound by uncoupled mitochondria and by mitochondria incubated under the resting state + MTX conditions represent the preprotein firmly associated with the *trans* site of the TOM complex or moving from the *trans* site of the TOM complex to the TIM complex while a band corresponding to the intermediate form of pb_2-DHFR means the fraction of the preprotein arrested simultaneously within the TOM and TIM complexes. Taking into account the amount of bound pb_2-DHFR, one could conclude that under the *trans* site binding conditions, the binding proceeds at remarkably higher levels than under the resting state + MTX conditions, which can be explain by excess of the TOM complex over the TIM complex (Dekker *et al.*, 1997; Sirrenberg *et al.*, 1997).

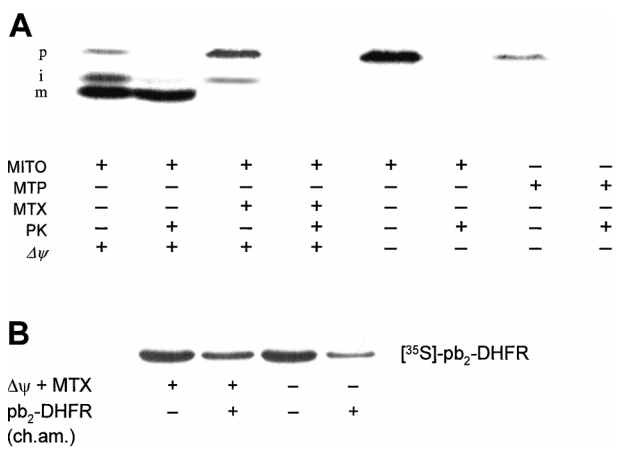

Fig. 1. Arresting of pb_2-DHFR within the TOM complex of yeast mitochondria. (A) pb_2-DHFR (3 μg per 50 μg of mitochondrial protein) was incubated with coupled ($+\Delta\psi$) and uncoupled ($-\Delta\psi$) yeast mitochondria (MITO) or mitoplasts (MTP) as described in the section Materials and Methods. For accumulation of pb_2-DHFR within both mitochondrial membranes, the preprotein was preincubated for 15 min on ice in the presence of 2 μM methotrexate before coupled mitochondria were added (MITO + MTX). External NADH, at concentration of 200 nmoles per 0.1 mg of mitochondrial protein, was applied as the respiratory substrate. After incubation samples were washed with HS buffer and reisolated (10 min, 12,000 $\times$ *g*). The pellets were resuspended in SM buffer and divided in two. One aliquot was treated with proteinase K (250 μg/ml, 10 min at 0°C) (+PK) while the second one was left untreated (−PK). Next, mitochondria or mitoplasts were reisolated, obtained pellets were subjected to SDS-PAGE, and bound pb_2-DHFR was visualized by immunoblotting with anti-mouse DHFR antiserum. (B) Mitochondria preincubated with chemical amounts of pb_2-DHFR (3 μg per 50 μg of mitochondrial protein) under the *trans* site binding ($-\Delta\psi$, −MTX) and the resting state + MTX ($+\Delta\psi$, +MTX) conditions were used to perform [^{35}S]pb_2-DHFR binding under the *trans* site binding conditions (see Materials and Methods). After the binding reaction samples were treated as in (A) and bound [^{35}S]pb_2-DHFR was visualized by fluorography. The levels of pb_2-DHFR and [^{35}S]pb_2-DHFR binding varied by not more than 14% in various experiments. p, i, and m, precursor, intermediate, and mature forms of pb_2-DHFR, respectively; ch. am., chemical amounts.

To check weather the above interactions of the precursor form of the applied preprotein with the TOM complex indeed caused the TOM complex blockage, we used mitochondria preincubated under the *trans* site binding and the resting state + MTX conditions in the presence or absence of chemical amounts of pb_2-DHFR (3 μg per 50 μg of mitochondrial protein) to perform the *trans* site binding for [^{35}S]pb_2-DHFR, i.e., pb_2-DHFR synthesized by *in vitro* transcription and translation in reticulocyte lysate in the presence of radioactive [^{35}S]methionine. Mitochondria preincubated in the presence of chemical amounts of pb_2-DHFR bound distinctly smaller amounts of the radiolabeled form of the preprotein at the *trans* site of the TOM complex than control mitochondria preincubated under the same conditions, but in the absence of pb_2-DHFR (Fig. 1B). Further, higher amounts

of [^{35}S]pb$_2$-DHFR were bound after preincubation under the resting state + MTX conditions. Thus, pb$_2$-DHFR bound under the *trans* site binding conditions restricted the permeability of the TOM complex considerably and more efficiently than after binding under the resting state + MTX conditions. Summing up, pb$_2$-DHFR bound by yeast uncoupled or coupled mitochondria, i.e., under the *trans* site binding or the resting state + MTX conditions, respectively, blocks the TOM complex channel, although at different levels.

We next investigated whether the blockage of the TOM complex channel would influence the transport of external NADH to the intermembrane space where the proper dehydrogenase is located on the surface of the inner membrane. In this case, oxidation of external NADH should be inhibited in the presence of pb$_2$-DHFR. To test this experimentally, isolated yeast mitochondria were preincubated with chemical amounts of pb$_2$-DHFR (3 μg per 50 μg of mitochondrial protein) under the *trans* site binding or the resting state + MTX conditions and oxidation of different concentrations of external NADH was recorded spectrophotometrically. In the case of mitochondria preincubated with pb$_2$-DHFR under the resting state + MTX conditions (coupled mitochondria), no inhibition of external NADH oxidation was observed. However, with mitochondria preincubated with the preprotein under the *trans* site binding conditions (uncoupled mitochondria), external NADH oxidation was inhibited, providing the substrate was applied in higher concentrations. As shown in Fig. 2A, when external NADH was applied at concentrations lower than 50 nmoles per 0.1 mg of mitochondrial protein, the blockage of the TOM complex

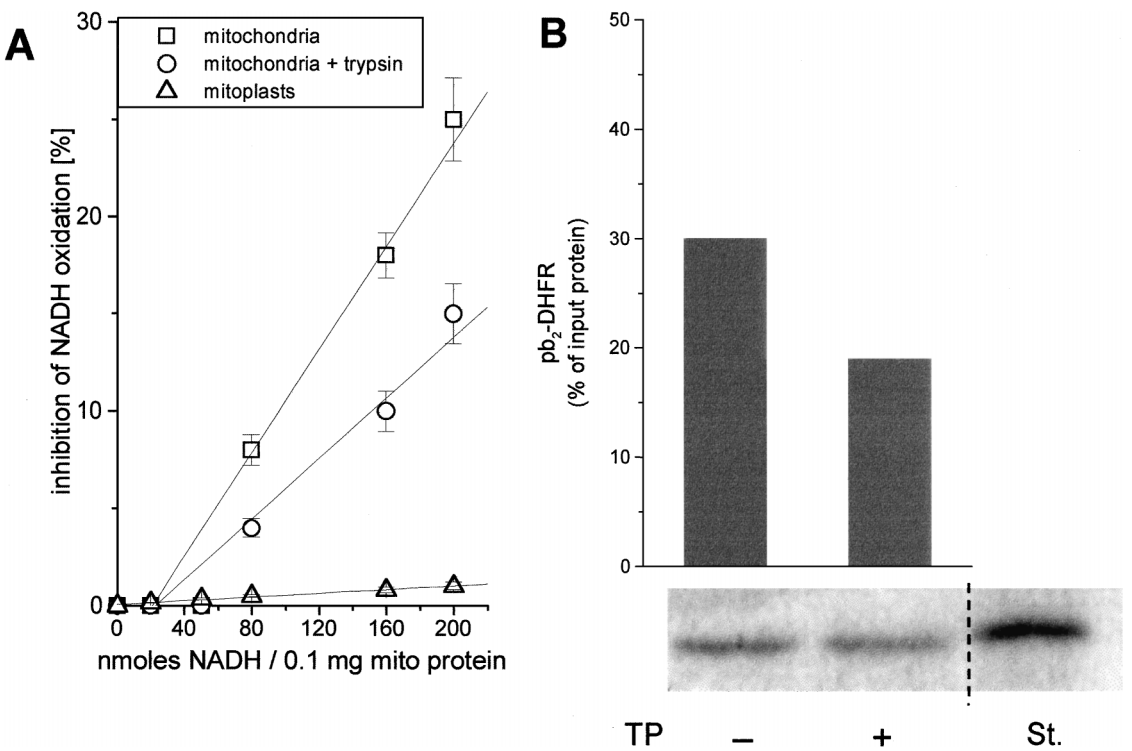

Fig. 2. Influence of pb$_2$-DHFR binding at the *trans* site of the TOM complex on oxidation of external NADH increasing concentrations by yeast uncoupled mitochondria. (**A**) The effect of trypsin pretreatment on inhibition of external NADH oxidation imposed by pb$_2$-DHFR binding at the *trans* site of the TOM complex. Native or trypsin-pretreated mitochondria, as well as mitoplasts, were incubated as for the *trans* site binding reaction (see Materials and Methods) and the rate of oxidation of different concentrations of external NADH was recorded spectrophotometrically. The data are mean values ± SEM of five different experiments. (**B**) The effect of trypsin pretreatment on the TOM complex activity. Trypsin pretreated (+TP) and control (−TP) mitochondria were incubated in the presence of [^{35}S]pb$_2$-DHFR under conditions enabling the *trans* site binding reaction. After incubation samples were washed with HS buffer and reisolated (10 min, 12,000 $\times g$)Π. Fractions of [^{35}S]pb$_2$-DHFR representing binding at the *trans* site of the TOM complex were visualized by fluorography and quantified by ScanPack 3.0. The rightmost lane contains 50% of input preprotein as standard (St.). Binding of [^{35}S]pb$_2$-DHFR at the *trans* site of the TOM complex or its import into mitochondria varied by not more than 15% in various experiments.

channel did not influence its rates of oxidation by yeast mitochondria. However, with higher concentrations of external NADH, the blockage of the TOM complex channel resulted in an increasing inhibition of the substrate oxidation. For example, in the presence of 200 nmoles of external NADH per 0.1 mg of mitochondrial protein, its oxidation was inhibited by about 25%. When mitoplasts were preincubated with the same amounts of pb_2-DHFR under the *trans* site binding conditions, inhibition of external NADH oxidation was not obtained. Thus, pb_2-DHFR can inhibit external NADH oxidation under the *trans* site binding conditions whereas under the resting state + MTX conditions, inhibition does not occur. This may result from the higher levels of the preprotein binding at the TOM complex in uncoupled mitochondria (see Fig. 1), as well as from the faster transport of external NADH to the intermembrane space of the mitochondria caused by the higher rate of external NADH oxidation resulting from dissipation of $\Delta\psi$. Since the inhibition appeared when external NADH was applied at concentrations higher than 50 nmoles per 0.1 mg of mitochondrial protein, one could conclude that in the presence of higher amounts of the substrate, its transport across the outer membrane may be supplemented by the TOM complex channel.

To test weather the inhibition of external NADH oxidation observed with uncoupled mitochondria was really due to the TOM complex blockage by pb_2-DHFR, we performed the same assay, but for trypsin-pretreated mitochondria. According to published data, preprotein binding to the *trans* site of the TOM complex is significantly slowed by removal of the surface receptors of the complex by trypsin pretreatment (Rapaport *et al.*, 1998) since the receptors are important for preprotein recognition and their subsequent translocation. Taking the observation into account, we assumed that removal of the surface receptors would weaken the inhibition of external NADH oxidation observed in the presence of pb_2-DHFR, if the latter resulted from the blockage of the TOM complex channel by pb_2-DHFR. To test this experimentally, isolated mitochondria were treated with trypsin prior to the binding reaction of pb_2-DHFR (3 μg per 50 μg of mitochondrial protein) and oxidation of external NADH was recorded spectrophotometrically. It is important to note, from Fig. 2B, that the applied trypsin pretreatment indeed weakened [^{35}S]pb_2-DHFR binding at the *trans* site of the TOM complex. In the case of trypsin-pretreated mitochondria (+TP), the binding was decreased by about 40% when compared to control mitochondria (−TP). This indicates that removal of the surface receptors hinders pb_2-DHFR transfer to the *trans* site of the TOM complex. As shown in Fig. 2A, the removal of the surface receptors also resulted in a weakened inhibition of external NADH oxidation by pb_2-DHFR incubated with mitochondria under the *trans* site binding conditions. For example, when external NADH was applied at concentration of 200 nmoles per 0.1 mg mitochondrial protein, the inhibition was decreased by about 40%. It should be emphasized here that neither pb_2-DHFR nor trypsin had any effect on VDAC1 (porin1) reconstituted into planar phospholipid membranes (not shown; see also Thieffry *et al.*, 1994). Thus, the effect of pb_2-DHFR on oxidation of external NADH at concentrations higher than 50 nmoles per 0.1 mg of mitochondrial protein appears to be specifically connected with the blockage of the TOM complex channel.

It is well known that the major pathway for external NADH transport across the outer membrane of yeast mitochondria is constituted by VDAC1 (Lee *et al.*, 1998). However, our results allow the conclusion that in yeast uncoupled mitochondria, the permeability of VDAC1 to external NADH is probably limited when the substrate is present in higher concentrations. This observation is in agreement with our previous finding that VDAC1 could restrict the traffic of external NADH through the outer membrane of yeast mitochondria under conditions leading to its faster transport through the membrane, i.e., in the uncoupled state obtained due to dissipation of $\Delta\psi$ (Kmita *et al.*, 1999). It has been recently reported (Kmita and Budzińska, 2000) that the TOM complex plays an important role in transport of external NADH into yeast mitochondria depleted of VDAC1. The inhibitory effect of the TOM complex blockage on oxidation of external NADH (applied at concentrations higher than 50 nmoles per 0.1 mg of mitochondrial protein) by wild-type uncoupled mitochondria suggests that, even in the presence of functional VDAC1, some amounts of external NADH might cross the outer membrane due to the TOM complex channel. In this case, one could expect pb_2-DHFR to compete with external NADH applied at higher concentrations for the transport through the outer membrane. Since, for chemical amounts of pb_2-DHFR, the competition was not observed (see also Kmita and Budzińska, 2000), [^{35}S]pb_2-DHFR was used. The binding reaction was performed by using isolated uncoupled mitochondria and binding of [^{35}S]pb_2-DHFR at the *trans* sites of the TOM complex was assessed by washing of the samples under high salt conditions (120 mM KCl). As shown in Fig. 3, an increase in external NADH concentrations slightly decreased amounts of bound [^{35}S]pb_2-DHFR. The largest amount of [^{35}S]pb_2-DHFR was bound in the absence of external NADH. Applying of external NADH at concentrations of 80 and 200 nmoles per 0.1 mg of mitochondrial protein resulted in a decrease of [^{35}S]pb_2-DHFR levels of binding by about 4 and 10%, respectively. Thus, increasing concentrations of external NADH slightly restrict the

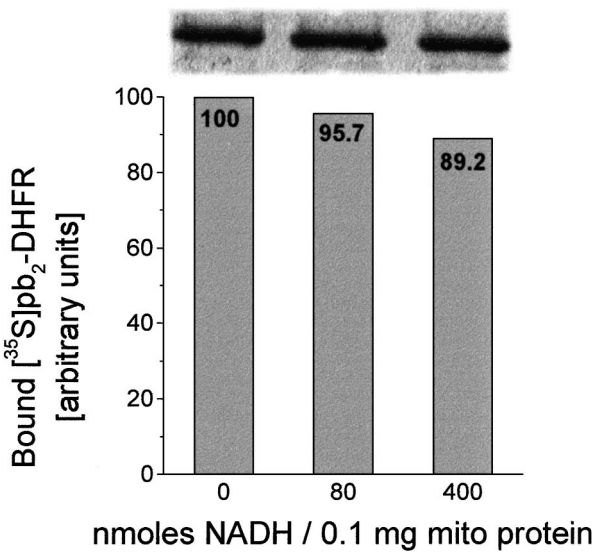

Fig. 3. Binding of [^{35}S]pb$_2$-DHFR to the *trans* site of the TOM complex of yeast uncoupled mitochondria in the absence and in the presence of external NADH. Mitochondria were incubated in the presence of [^{35}S]pb$_2$-DHFR under the *trans* site binding conditions (see Materials and Methods) in the absence of external NADH or in the presence of the substrate at the concentrations of 80 and 200 nmoles per 0.1 mg of mitochondrial protein. After washing with HS buffer, samples were separated by SDS-PAGE. Bound [^{35}S]pb$_2$-DHFR was visualized by fluorography and quantified by ScanPack 3.0. Binding of [^{35}S]pb$_2$-DHFR varied by not more than 10% in various experiments.

access of the applied preprotein to the *trans* site of the TOM complex, which could be due to the transport of a fraction of the substrate by the TOM complex. The fraction would increase with increasing concentrations of external NADH.

It has been concluded by Xu *et al.* (1999) that the probability of VDAC channels being open in isolated mitochondria varies with the particular isoform displaying channel-forming activity. Further, VDAC closures are known to be facilitated by external NADH (Lee *et al.*, 1994; Zizi *et al.*, 1994). The *S. cerevisiae* mitochondria contain only one VDAC isoform able to form a channel (VDAC1). Our results allow the conclusion that, under conditions of its limited permeability, transport of metabolites across the outer membrane may be supplemented by the protein import channel within the TOM complex.

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REFERENCES

Benz, R. (1994). *Biochim. Biophys. Acta* **1197**, 167–196.
Benz, R., Janko, K., Boos, W., and Lauger, P. (1978). *Biochim. Biophys. Acta* **511**, 305–319.
Blachly-Dyson, E., Zambrowicz, E. B., Yu., W. H., Adams, V., McCabe, E. R. B., Adelman, J., Colombini, M., and Forte, M. (1993). *J. Biol. Chem.* **268**, 1835–1841.
Blachly-Dyson, E., Song, J., Wolfgang, W. J., Colombini, M., and Forte, M. (1997). *Mol. Cell. Biol.* **17**, 5727–5738.
Colombini, M. (1979). *Nature* (*London*) **279**, 643–645.
Colombini, M., Blachly-Dyson, E., and Forte, M. (1996). In *Ion Channels: VDAC, A Channel in the Outer Mitochondrial Membrane* (Narahashi, T., ed.), Plenum Press, New York, pp. 169–201.
Daum, G., Bohni, P. C., and Schatz, G. (1982). *J. Biol. Chem.* **257**, 13028–13033.
Dekker, P. J., Martin, F., Maarse, A. C., Bümer, U., Müller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997). *EMBO J.* **16**, 5408–5419.
De Pinto, V., Ludwig, O., Krause, J., Benz, R., and Palmieri, F. (1987). *Biochim. Biophys. Acta* **894**, 109–119.
De Vries, S. and Marres, C. A. M. (1987). *Biochim. Biophys. Acta* **895**, 205–239.
Dihanich, M., Suda, K., and Schatz, G. (1987). *EMBO J.* **6**, 723–728.
Dihanich, M., Schmid, A., Oppliger, W., and Benz, R. (1989). *Eur. J. Biochem.* **181**, 703–708.
Douce, R., Bourguignon, J., Brouquisse, R., and Neuberger, M. (1984). *Methods Enzymol.* **148**, 403–415.
Elkeles, A., Breiman, A., and Zizi, M. (1997). *J. Biol. Chem.* **272**, 6252–6260.
Fevre, F., Chich, J. F., Lauquin, G. J.-M., Henry, J. P., and Thieffry, M. (1990). *FEBS Lett.* **262**, 201–204.
Guo, X. J. and Lauquin, G. J.-M. (1986). *EBEC Rep.* **4**, 292.
Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R., and Pfanner, N. (1998). *Nature* (*London*) **395**, 516–521.
Juin, P., Thieffry, M., Henry, J.-P., and Vallette, F. M. (1997). *J. Biol. Chem.* **272**, 6044–6050.
Kamo, N., Maratsugu, M., Hongoh, R., and Kobatake, Y. J. (1979). *J. Membr. Biol.* **49**, 105–121.
Kmita, H., Stobienia, O., and Michejda, J. (1999). *Acta Biochim. Polon.* **46**, 991–1000.
Kmita, H. and Budzińska, M. (2000). *Biochim. Biophys. Acta* **1509**, 86–94.
Kuenkele, K.-P., Heins, S., Dembowski, M., Nargang, F. E., Benz, R., Thieffry, M., Walz, J., Lill, R., Nussberger, S., and Neupert, W. (1998a). *Cell* **93**, 1009–1019.
Kuenkele, K.-P., Juin, P., Pompa, C., Nargang, F. E., Henry, J.-P., Neupert, W., Lill, R., and Thieffry, M. (1998b). *J. Biol. Chem.* **273**, 31032–31039.
Lämmli, U. K. (1970). *Nature* (*London*) **227**, 680–685.
Lee, A. C., Zizi, M., and Colombini, M. (1994). *J. Biol. Chem.* **269**, 30974–30980.
Lee, A. C., Xu, X., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998). *J. Membr. Biol.* **161**, 173–181.
Lill, R. and Neupert, W. (1996). *Trends Cell Biol.* **6**, 56–61.
Mayer, A., Neupert, W., and Lill, R. (1995). *Cell* **80**, 127–137.
Michejda, J., Guo, X. J., and Lauquin, G. J.-M. (1990). *Biochem. Biophys. Res. Commun.* **171**, 354–361.
Michejda, J., Kmita, H., Stobienia, O., Budzińska, M., and Lauquin, G. J.-M. (1994). In *Molecular Biology of Mitochondrial Transport System: Restriction of Metabolite Permeation through the Outer Mitochondrial Membrane of Porin-Deficient Yeast Mutant*

(Forte, M. and Colombini, M., eds.), Springer-Verlag, Heidelberg, pp. 341–356.

Rapaport, D., Neupert, W., and Lill, R. (1997). *J. Biol. Chem.* **272**, 18725–18731.

Rapaport, D., Mayer, A., Neupert, W., and Lill, R. (1998). *J. Biol. Chem.* **273**, 8806–8813.

Sampson, M. J., Lovell, R. S., and Craigen, W. J. (1997). *J. Biol. Chem.* **272**, 18966–18973.

Schein, S. J., Colombini, M., and Finkelstein, A. V. (1976). *J. Membr. Biol.* **30**, 99–120.

Sirrenberg, Ch., Endres, M., Becker, K., Bauer, M. F., Walther, E., Neupert, W., and Brunner, M. (1997). *J. Biol. Chem.* **272**, 29963–29966.

Tarassov, I., Entelis, N., and Martin, R. P. (1995). *J. Mol. Biol.* **245**, 315–323.

Thieffry, M., Chich, J. F., Goldschmidt, D., and Henry, J. P. (1988). *EMBO J.* **7**, 1449–1454.

Thieffry, M., Fevre, F., Pelleschi, M., and Henry, J.-P. (1994). In *Molecular Biology of Mitochondrial Transport Systems: The Mitochondrial Outer Membrane Contains at Least Two Distinct Channels* (Forte, M. and Colombini, M., eds.), Springer-Verlag, Berlin.

Vallette, F. M., Juin, P., Peleschi, M., and Henry, J.-P. (1994). *J. Biol. Chem.* **269**, 13367–13374.

Voos, W., Gambill, B. D., Laloraya, S., Ang, D., Graig, E. A., and Pfenner, N. (1994). *Mol. Cell. Biol.* **14**, 6627–6634.

Wienhues, U., Koll, H., Becker, K., Guiard, B., and Hartl, F.-U. (1992). In *Protein Targeting. Practical Approach: Protein Targeting to Mitochondria* (Magge, A. and Wileman, T., eds.), Oxford University Press, London pp. 136–159.

Xu, X., Decker, W., Sampson, M. J., Craigen, W. J., and Colombini, M. (1999). *J. Membr. Biol.* **170**, 89–102.

Zizi, M., Forte, M., Blachly-Dyson, E., and Colombini, M. (1994). *J. Biol. Chem.* **269**, 1614–1616.