J. Membrane Biol. 161, 173–181 (1998)

The Journal of
Membrane
Biology

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The Role of Yeast VDAC Genes on the Permeability of the Mitochondrial Outer Membrane

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Received: 23 May 1997/Revised: 3 October 1997

Abstract. In addition to the *POR1* gene, which encodes the well-characterized voltage dependent anion-selective channel (YVDAC1) of the mitochondrial outer membrane, the yeast *Saccharomyces cerevisiae* contains a second gene (*POR2*) encoding a protein (YVDAC2) with 50% sequence identity to YVDAC1. Mitochondria isolated from yeast cells deleted for the *POR1* gene (Δ*por1*) had a profoundly reduced outer membrane permeability as measured by the ability of an intermembrane space dehydrogenase to oxidize exogenously added NADH. Mitochondria missing either YVDAC1 or both YVDAC1 and YVDAC2 showed a 2-fold increase in the rate of NADH oxidation when the outer membrane was deliberately damaged. Mitochondria from parental cells showed only a 10% increase indicating that the outer membrane is highly permeable to NADH. In the absence of YVDAC1, we calculate that the outer membrane permeability to NADH is reduced 20-fold. The low NADH permeability in the presence of YVDAC2 was not due to the low levels of YVDAC2 expression as mitochondria from cells expressing levels of YVDAC2 comparable to those of YVDAC1 in parental cells showed no substantial increase in NADH permeability, indicating a minimal role of YVDAC2 in this permeability. The residual permeability may be due to other pathways because cells missing both genes can still grow on nonfermentable carbon sources. However, YVDAC1 is clearly the major pathway for NADH flux through the outer membrane in these mitochondria.

Key words: VDAC — Mitochondria — Outer membrane — Dehydrogenase — NADH

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Introduction

The metabolite flux between the cytosol and the mitochondrial space is essential to mitochondrial function and hence to life for most eucaryotes. VDAC, a major protein of the mitochondrial outer membrane, forms large (diameter 2.5–3 nm) voltage-gated pores when introduced into planar phospholipid membranes. Similar channels are formed by VDAC in the outer membrane and these are thought to provide the major pathway for metabolite flux through the outer membrane (Colombini, 1979; Mannella, Forte, & Colombini, 1992; Mannella & Colombini, 1984). Treatment of VDAC channels in planar membranes with a variety of physiological and nonphysiological agents increases the probability of channel closure, and these agents have been shown to greatly inhibit function in isolated mitochondria by inhibiting the flux of metabolites through the mitochondrial outer membranes (the VDAC modulator: Holden & Colombini, 1988; Liu & Colombini, 1992; König's polyanion: Colombini et al., 1987; Benz et al., 1988; osmotic pressure: Zimmerberg & Parsegian, 1986; Gellerich et al., 1993; NADH: Zizi et al., 1994; Lee, Zizi & Colombini, 1994).

The yeast *Saccharomyces cerevisiae* contains two genes encoding VDAC proteins. The *POR1* gene encodes the YVDAC1 protein, which has been extensively characterized after reconstitution into planar phospholipid membranes. The recently identified *POR2* gene encodes a second protein (YVDAC2) with about 50% sequence identity to YVDAC1 (Forte, Blachly-Dyson & Colombini, 1996; Blachly-Dyson et al., 1997). If VDAC does provide the major permeability pathway of the mitochondrial outer membrane, then cells lacking the VDAC genes should be severely defective for respiration. Yeast with defects in respiration fails to grow on media containing only nonfermentable carbon sources

such as glycerol or lactate. When the *POR1* gene in yeast was deleted, the cells were able to grow on glycerol after a lag phase (Dihanich, Suda & Schatz, 1987) at 30°C but not at 37°C (Blachly-Dyson et al., 1990). Cells missing both the *POR1* and *POR2* genes grew on glycerol at rates similar to those just missing YVDAC1 (Forte et al., 1996) indicating the existence of another pathway for metabolite flux through the outer membrane. When the YVDAC2 protein is overexpressed in cells lacking YVDAC1, it restores the ability of the cells to grow on glycerol at 37°C (Forte et al., 1996; Blachly-Dyson et al., 1997). However, YVDAC2 has not been demonstrated to form channels. Thus, the importance of VDAC in metabolite flux through the outer membranes needs to be assessed in a more direct way.

Since, to our knowledge, no one had reported a method for measuring the permeability of the outer membrane in intact yeast mitochondria, we developed one by taking advantage of the very active NADH dehydrogenase in the yeast mitochondrial intermembrane space. Using this method on mitochondria isolated from cells expressing both VDAC genes or cells missing one or both VDAC genes, we found large differences in the permeability which indicate that YVDAC1 is the major pathway for NADH flux through the outer membrane.

Materials and Methods

Yeast Strains

Yeast strain M22-2 (Δ*por1*), has been previously described (Blachly-Dyson et al., 1990). M3 is the *POR1* parent of M22-2. M22-2-1 (Δ*por1* Δ*por2*) was constructed from M22-2 by gene transplacement to delete the coding region of the *POR2* gene. Strain *M-V1PV2* was constructed from M22-2 by using homologous recombination to insert a construct encoding YVDAC2 under the control of the *POR1* promoter at the *POR1* locus. This strain overexpressed YVDAC2 in that it produced YVDAC2 levels similar to the YVDAC1 levels in the parent M3 (Blachly-Dyson et al., 1997).

Preparation of Yeast Cells

To control the cell concentration, a stock solution of cells was prepared. A colony of yeast cells was inoculated into 100 ml of medium which contains 0.3 g of yeast extract, 0.1 g of potassium phosphate, 0.1 g of NH_4Cl, 0.05 g of $CaCl_2$, 0.05 g of NaCl, 0.06 g of $MgSO_4$, 0.03 ml of 1% $FeCl_3$ and 2.4 ml of 85% lactic acid. The medium was adjusted to pH 5.0 by adding solid KOH. When the cells reached an O.D. of between 0.6 and 0.8 (at 600 nm) they were stored at 4°C for later use. The stocks of the mutant cell lines were recultured once a month. For mitochondrial isolation, 9 ml of parental yeast stock solution, 6 ml of yeast mutant missing YVDAC1 gene, or 60 ml of yeast mutant missing both genes was inoculated into one liter of the same medium and grown with shaking at 30°C. An O.D. between 0.5 and 0.6 was reached at 24 hr (parental yeast), 38 hr (Δ*por1* yeast) and 48 hr (Δ*por1* Δ*por2* yeast) after inoculation. Typically, four liters of cell suspension were harvested when the culture reached late log phase (O.D. between 0.5 and 0.6). Six grams of cells were obtained.

The Isolation of Intact Mitochondria

The isolation procedure is essentially that described by Daum et al. (1982). However, important modifications were made in order to achieve high levels of outer membrane intactness. The yeast cells were pelleted, washed once with distilled water, and suspended in 0.1 M $Tris.SO_4$, 10 mM DTT, pH 9.4 at 0.5 g wet weight cells per 1 ml of medium. After incubation at 30°C for 10 min, the cells were pelleted, washed once with 1.2 M sorbitol, and resuspended in 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.2. Zymolyase (20,000 U/g) (Seikagaku Corporation, Ijamsville, MD) was added at 10 mg per gram wet weight of cells and the suspension was incubated at 30°C for 25 min to allow digestion of the cell walls. Dilution into 10% N-lauroylsarcosine was used to check for spheroplast formation. The spheroplasts were harvested at 6000 rpm for 5 min. (GSA rotor) and washed twice with the 1.2 M sorbitol solution. Washing did not involve resuspension of the spheroplasts but rotation of the centrifuge bottle by 180°C (about its long axis) and recentrifugation after adding the fresh buffer. The washed spheroplasts were then suspended in 60 ml of H-medium containing 0.6 M mannitol, 10 mM Tris.Cl, 0.6% PVP, 0.1 mM EGTA and 0.1% BSA, pH 7.2, by using a Pipetman P5000 with 1 cm cutoff the disposable tip. No homogenization was used. This mild method of lysing the spheroplasts allowed us to obtain a high level of intact mitochondria (intactness between 88% and 100%). The suspension was centrifuged at 3000 rpm for 5 min (SS-34 rotor) and the mitochondrion-containing supernatant was collected. The pellet was suspended again by the same procedure and recentrifuged. Again the supernatant was saved. Two ml of 10% of Percoll was layered at the bottom of each of the two supernatants to keep the pellets loose. The tubes were centrifuged at 9,000 rpm (SS-34 rotor) for 10 min. The pellets were suspended in H-medium, pooled, and centrifuged at 3,000 rpm for 5 min. The supernatant was again centrifuged at 9,000 rpm for 10 min over a 2 ml layer of 10% Percoll. The pellet was suspended in a small volume of H-medium (4 to 6 ml). This suspension was layered on the top of a Percoll gradient (14 ml of 45%, 8 ml of 21% and 6 ml of 5%) and centrifuged at 10,500 rpm for 30 min. The major band in the Percoll gradient was obtained, washed with H-medium once and suspended in 2 ml of H-medium.

A portion of mitochondrial suspension was diluted 10-fold with R-medium containing 0.65 M sucrose, 10 mM HEPES, 10 mM potassium phosphate, 5 mM KCl and 5 mM $MgCl_2$ pH 7.2 in order to measure NADH oxidation. Another portion was hypotonically shocked, as follows, to break the mitochondrial outer membrane. It was incubated with 2 volumes of distilled water for 10 min on ice followed by the addition of 5 volumes of R-medium. Finally, 2 volumes of 2X R-medium were added to return the osmotic pressure to normal. This order of addition minimized damage to the inner membrane.

The Measurement of Outer Membrane Intactness

The intactness of the mitochondrial outer membrane was quantitated by measuring cytochrome c-dependent oxygen consumption (Douce et al., 1987). Exogenously added reduced cytochrome c must pass through the outer membrane to be oxidized by the cytochrome c oxidase on the outer surface of the inner membrane. The percent intactness of the mitochondrial outer membrane for each preparation was calculated as follows:

$$\%\ intactness = \left(1 - \frac{v_{intact}}{v_{disrupted}}\right) \cdot 100$$

where v_{intact} and $v_{\text{disrupted}}$ are the KCN-sensitive rate of oxygen consumption of intact and hypotonically disrupted mitochondria, respec-

tively. The mitochondria were disrupted with a severe osmotic shock by mixing 40 μl of mitochondrial suspension with 1.5 ml water. After 3 min, 1.5 ml of 2 times concentrated respiration buffer was added to restore isoosmotic conditions. For these experiments, we used a mitochondrial protein concentration of 50–80 μg/ml.

The Assay of Protein Content

Mitochondrial protein was measured using the BCA method (Pierce, Rockford, IL) following addition of Triton X-100 to the mitochondrial suspension (1% w/v final). Bovine serum albumin was the standard.

The Enzyme Activity Assays

Cytochrome c oxidase activity was measured as cyanide-sensitive oxygen consumption in the presence of cytochrome c and ascorbate. Mitochondria were disrupted to maximally expose cytochrome c oxidase activity. A severe osmotic shock was applied by adding 40 μl of mitochondrial suspension to 1.5 ml of distilled water and incubating for 3 min. To restore the original aqueous conditions, 1.5 ml of 2X R-medium was added. Oxygen consumption was monitored with a Clark oxygen electrode. Ascorbate was added (50 μl of 0.48 M) to maintain the cytochrome c reduced. The oxygen consumption was stimulated by the addition of 180 μg of cytochrome c. Finally, KCN (0.2 mM final) was used to inhibit the cytochrome c oxidase activity and to determine the level of any KCN-resistent respiration.

Fumarase activity was assayed by monitoring fumarate production at 250 nm (Robinson et al., 1987). The solution was buffered with 0.1 M potassium phosphate, pH 7.4 and 50 mM of malate was added to start the reaction. To obtain total fumarase, mitochondria were permeabilized by adding 1% of Triton X-100.

The NADH oxidation was measured by monitoring the decrease in absorbance at 340 nm (typically by recording at 5-sec intervals). Intact mitochondria and hypotonically shocked mitochondria were used as samples. About thirty micromolar NADH (final) was added to start the reaction. Uncouplers and benzoquinone were added to mitochondria and mixed well before the addition of NADH. All the uncouplers were dissolved in ethanol and stored at −20°C in the dark. Benzoquinone was dissolved in R-medium. The final concentration of ethanol in mitochondria was less than 0.5%. Control experiments showed that up to 2% ethanol has no effect on the NADH oxidation (*data not shown*).

Results and Discussion

Growth Rates of Parental and Mutant Yeast Cells

If VDAC proteins are important for mitochondrial respiration, then cells lacking VDAC should be impaired in their ability to grow on nonfermentable carbon sources, such as glycerol and lactate. Delayed or temperature-sensitive growth of Δ*por1* yeast on glycerol has previously been described (Dihanich et al., 1987; Blachly-Dyson et al., 1990; Michejda, Guo & Lauquin, 1990). We measured growth rates of parental, Δ*por1,* and Δ*por1* Δ*por2* yeast on lactate medium at 30°C (Fig. 1). The doubling times for growth of parental, Δ*por1,* and Δ*por1* Δ*por2* yeast under these conditions were 4, 5.5, and 11 hr, respectively. The ability of the Δ*por1* Δ*por2* cells to grow on lactate indicates that these cells must contain pathways other than VDAC for metabolite transport through the mitochondrial outer membrane. However, the much slower growth of the double mutant relative to Δ*por1* indicates that the presence of YVDAC2 contributes to the ability of cells to grow under these conditions. The sigmoidal growth curves for the VDAC mutants reflect a lag time in growth after transfer from glucose to lactate media, which may be similar to the lag time for growth on glycerol reported by Dihanich et al. (1987).

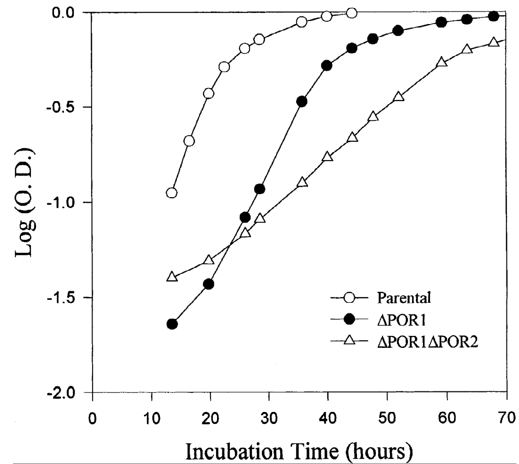

Fig. 1. The growth curves for parental yeast (open circle), Δ*por1* yeast (closed circle) and Δ*por1* Δ*por2* yeast (open triangle). The cell concentration was monitored by absorbance at 600 nm. The absorbance was log transformed and plotted as a function of time.

NADH Oxidation Rates and the Influence of the Outer Membrane

When NADH is added externally to isolated mitochondria, it is oxidized primarily by an NADH dehydrogenase located on the outer surface of the mitochondrial inner membrane (Ohnishi, Kawaguchi & Hagihara, 1966). If the outer membrane limits the flux of NADH, the rate of NADH oxidation would increase upon damage of the outer membrane. The lower the outer membrane permeability, the larger the increase in respiration upon damage of the outer membrane.

Figure 2*A* shows the rate of oxidation of externally added NADH by parental mitochondria, both intact and mitochondria whose outer membrane was damaged by mild hypotonic shock. After hypotonic shock, the rate of NADH oxidation was faster as indicated by a faster drop in NADH concentration with time (solid symbols). In hypotonically treated mitochondria, the NADH dehydrogenase is presumably in contact with the medium [NADH], while in the intact mitochondria the enzyme

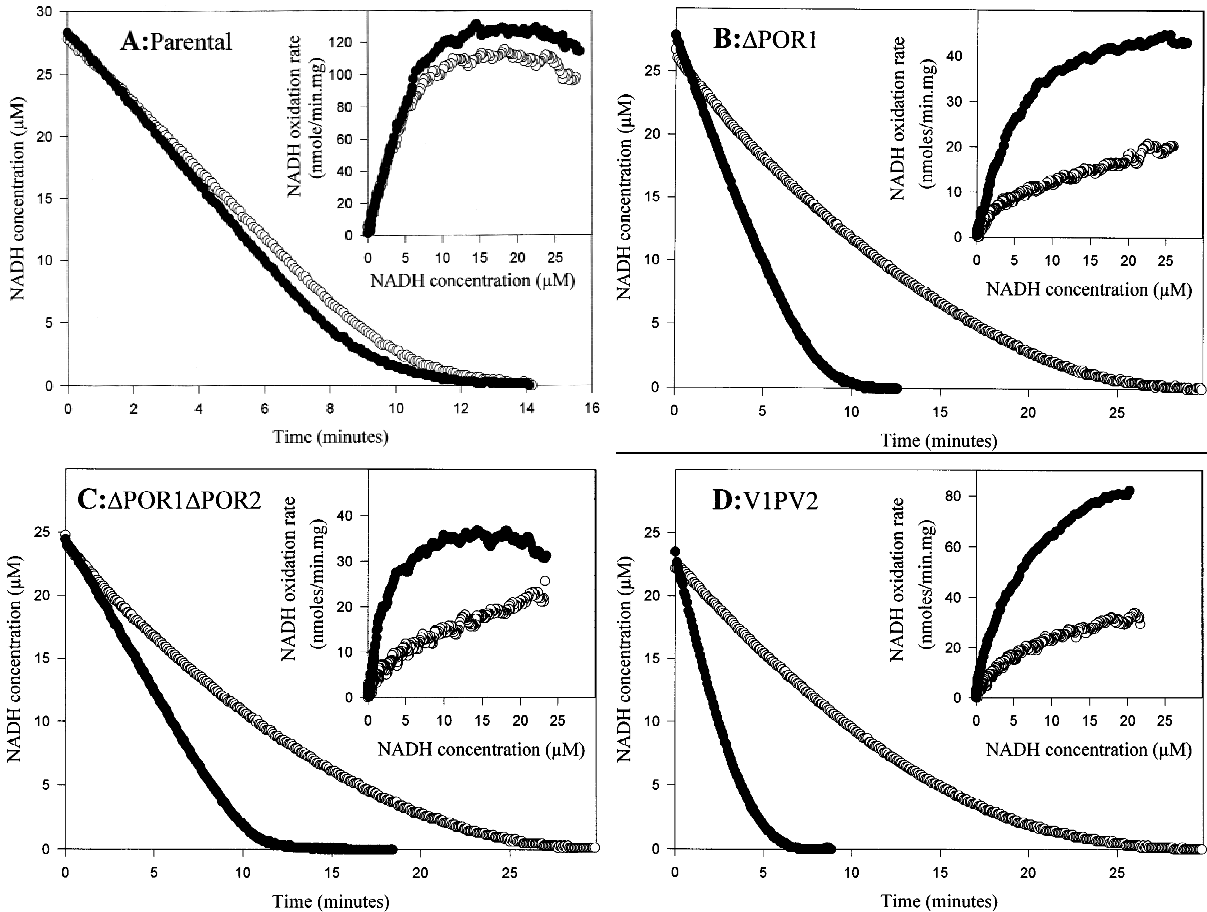

Fig. 2. NADH oxidation by mitochondria isolated from parental (*A*), Δ*por1* (*B*), Δ*por1* Δ*por2* (*C*), and *V1PV2* (*D*) yeast cells. The NADH oxidation was monitored by the decrease in absorbance at 340 nm. The absorbance was converted to NADH concentration by dividing it by the molar extinction coefficient (6.22×10^3). The data for intact and hypotonically shocked mitochondria are indicated by open circles and closed circles, respectively. The oxidation rate in inset was generated as described in the text and divided by the amount of mitochondrial protein added (25 μg for parental, 85 μg for Δ*por1,* and 70 μg for Δ*por1* Δ*por2* and *V1PV2*).

experiences a lower concentration of NADH, limited by the permeability of the outer membrane.

To obtain the oxidation rate (Fig. 2*A,* inset), linear regression was used on each group of 13 adjacent points. This yielded minimum noise without significant filtration of the signal. In the inset, the rate of oxidation at high [NADH] corresponds to the decay in [NADH] at early times (main figure). The rate is constant and independent of concentration at [NADH] above about 15 μM, but is lower for intact than for hypotonically treated mitochondria. This is probably because this dehydrogenase is activated by NADH, dependent on the initial concentration of NADH present in the assay (Møller, Johnston & Palmer, 1981) so that the apparent V_{max} increases with increase in the initial [NADH]. Since the dehydrogenase in mitochondria with damaged outer membranes experiences a higher initial [NADH] than that in intact mitochondria, the apparent V_{max} is higher.

The increase in the rate of NADH oxidation of hypotonically treated mitochondria is due only to the removal of the mitochondrial outer membrane, and not to release of enzymes from the mitochondrial matrix. Less than 3% of the fumarase leaked from the mitochondrial matrix during hypotonic shock treatment (*data not shown*). Thus the mitochondrial inner membrane was still largely intact after this mild treatment. Similar results were published by Daum et al. (1982). They reported that fumarase is not significantly released from the matrix even when the medium osmolality was reduced 6-fold to 0.1 osmolal.

In mitochondria from Δ*por1* cells, the difference in NADH oxidation rate between intact and hypotonically shocked mitochondria is much greater than for parental mitochondria. Figure 2*B* shows that 26 μM NADH is completely consumed within 10 min by Δ*por1* mitochondria with damaged outer membranes. By contrast, intact Δ*por1* mitochondria needed 26 min to oxidize this amount of NADH. The NADH oxidation rates (inset) of

hypotonically shocked Δ*por1* mitochondria are roughly twice those of intact mitochondria. Similar results were obtained for Δ*por1* Δ*por2* mitochondria whose outer membranes are missing both YVDAC1 and YVDAC2 (Fig. 2*C*). Since YVDAC2 is expressed at roughly 5–8-fold lower levels than YVDAC1, we examined the NADH permeability of mitochondria prepared from cells (called *V1PV2*) in which expression of the YVDAC2 protein is mediated by the *POR1* promoter, resulting in levels of YVDAC2 similar to that of YVDAC1 (Blachly-Dyson et al., 1997). The dramatic stimulation of dehydrogenase activity after damaging the outer membrane was still present (Fig. 2*D*). The permeability of mitochondria prepared from this strain is similar to Δ*por1* Δ*por2* mitochondria. This indicates that YVDAC2 may not confer significant permeability for NADH to the outer membrane.

The highest NADH dehydrogenase activities in mitochondria from parental yeast are 3–4 times higher than those in mitochondria from Δ*por1* and Δ*por1* Δ*por2* yeast (insets in Fig. 2). Higher levels were achieved in the mitochondria from *V1PV2* yeast but these were still less than the parental levels. This decrease on overall activity of the dehydrogenase may be due to different amounts of dehydrogenase expressed in the mutant cell lines or perhaps due to incomplete breakage of the outer membrane by hypotonic shock. The former interpretation is supported by the fact that CN^--sensitive cytochrome c oxidase activities of Δ*por1* and Δ*por1* Δ*por2* mitochondria are 65% and 85% of that of parental mitochondria, respectively (*data not shown*). The decrease in CN^--sensitive cytochrome c oxidase activity of Δ*por1* mitochondria is consistent with previous reports (Dihanich et al., 1987). The reason for the activity of the cytochrome c oxidase of Δ*por1* Δ*por2* mitochondria being greater than that of Δ*por1* mitochondria is unknown. In contrast, the fumarase activity of Δ*por1* mitochondria is not different from that found in mitochondria from parental cells while Δ*por1* Δ*por2* mitochondria showed a 20% reduction.

Determination of the Permeability of the Outer Membrane to NADH

Figures 2 and 3 show that the mitochondrial outer membranes lacking YVDAC1 are substantially less permeable than those containing YVDAC1. To quantify the permeabilities of the outer membrane to NADH, we modified the method developed to measure the permeability to ADP (Lee et al., 1994). The method of Lee et al. could not be used directly to determine the permeability of the mitochondrial outer membrane to NADH for two reasons: first, the NADH activation of the dehydrogenase that results in a variable Km of the enzyme, depends on the initial concentration of NADH (Møller et al., 1981), and second, the NADH inhibition of outer membrane permeability. Lee et al. (1994) observed a 6-fold reduction of the permeability of the mitochondrial outer membrane by NADH and attributed it to closure of VDAC channels (Zizi et al., 1994). Thus, the method of Lee et al., which assumes constant permeability with time, is not appropriate because the [NADH] decays with time during the oxidation measurements with resulting changes in permeability.

The results illustrated in the insets in Fig. 2 show that the rate of oxidation of a given medium [NADH] is very different for intact and shocked mitochondria, as would be expected if the outer membrane imposes a permeability barrier between the medium and the enzyme. A steady state is established between the NADH flux through the outer membrane and the NADH oxidation in the intermembrane space. Thus the NET flux across the outer membrane is equal to the oxidation rate.

$$\begin{aligned} \textit{rate of NADH oxidation} &\\ &= \textit{net NADH flux across outer membrane} \\ &= P\,([NADH]_{out} - [NADH]_{in}) \end{aligned}$$

The permeability of the membrane was determined by taking the rate of NADH oxidation for intact mitochondria as the flux of NADH through the outer membrane and dividing it by the concentration difference of NADH across the membrane. The medium [NADH] was directly determined from the absorbance measurements. The intermembrane space [NADH] was estimated by comparing the rates of oxidation observed with intact and hypotonically treated mitochondria. We assumed that in the shocked mitochondria, the [NADH] in the medium is the same as in the intermembrane space.

To calculate the [NADH] in the intermembrane space of the intact mitochondria, we reasoned that the same [NADH] at the enzyme should produce the same oxidation rate. Thus, by determining the [NADH] necessary to cause a particular oxidation rate in shocked mitochondria, we can determine the [NADH] in the intermembrane space of intact mitochondria that are oxidizing NADH at the same rate. This method has the virtue of not assuming any particular type of kinetics such as Michaelis-Menten. It simply generates a relationship between [NADH] and oxidation rate by using the shocked mitochondria and applies this information to the intact mitochondria. The digitized data collected from the shocked mitochondria contained only a finite number of points and some level of noise and thus we fitted the data to a 9th-order polynomial in order to obtain a function that embodies how oxidation rate depended on [NADH]. To use this function to obtain values of the [NADH] for particular values of the oxidation rate (measured with intact mitochondria), we applied Newton's approximation.

Once the intermembrane space [NADH] was deter-

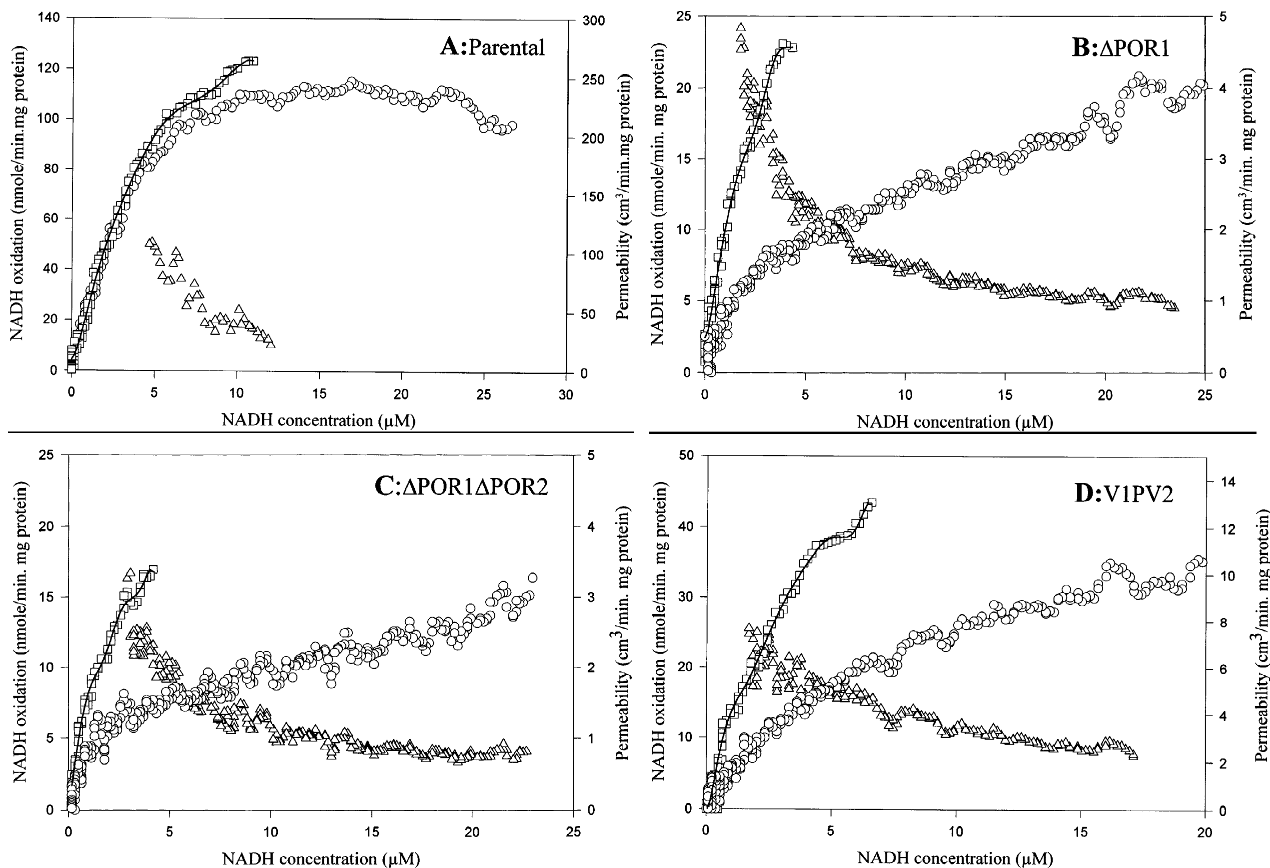

Fig. 3. NADH oxidation rates and the outer membrane permeabilities as a function of [NADH]. The data were obtained as described in Fig. 2. The oxidation rates of intact and hypotonically shocked mitochondria are indicated by circles and squares, respectively. The solid line is the ninth-power polynomial fit of the data from hypotonically shocked mitochondria. The open triangles indicate the permeability of the mitochondrial outer membrane calculated from the oxidation rates. The panels illustrate results for mitochondria isolated from parental, Δ*por1,* Δ*por1* Δ*por2,* and *V1PV2* yeast cells. The amount of mitochondrial protein used for each assay was 25 μg for parental, 85 μg for Δ*por1,* 91 μg for Δ*por1* Δ*por2,* and 49 μg for *V1PV2.*

mined from each value of the oxidation rate recorded using intact mitochondria (in experiments as in Fig. 2), the permeability was easily calculated. Each value of the intermembrane space [NADH] was subtracted from the corresponding medium [NADH] measured at the same time and thus the concentration difference was obtained. The oxidation rate divided by the corresponding concentration difference yields the permeability. This is the total permeability (not permeability per unit area) and hence the units are cm^3/min. This was normalized by the protein content. In this way, a set of permeability values was generated. At low NADH concentrations (below 2–3 μM) the concentration differences were insignificant compared to the noise and the calculated permeability values are not meaningful.

To avoid different degrees of dehydrogenase activation by initial NADH levels, experiments were designed to produce the same initial NADH oxidation rate in intact and broken mitochondria (and thus presumably the same concentration of NADH at the enzyme) by adding different concentrations of NADH to the solution. Figure 3 shows typical results along with the calculated outer membrane permeability values (triangles).

For mitochondria isolated from the parental cells, the calculated permeability varies with [NADH]. This variation is attributed to closure of VDAC channels in mitochondria from parental cells due to the previously demonstrated effect of β-NADH on VDAC (Zizi et al., 1994; Lee et al., 1994). However this does not explain the smaller but reproducible dependence of permeability on [NADH] observed with mitochondria lacking both VDAC proteins. The variation does not seem to arise from some artifact of the method.

The value of the outer membrane permeability is most affected by the loss of YVDAC1. In the parental, the permeability ranges between 20 and 120 cm^3/min · mg protein, depending on the [NADH]. This is roughly 20 times the value found for mitochondria from the mutant cells. In Δ*por1* it drops to 1 to 5 cm^3/min · mg protein. This is similar to that found for Δ*por1*

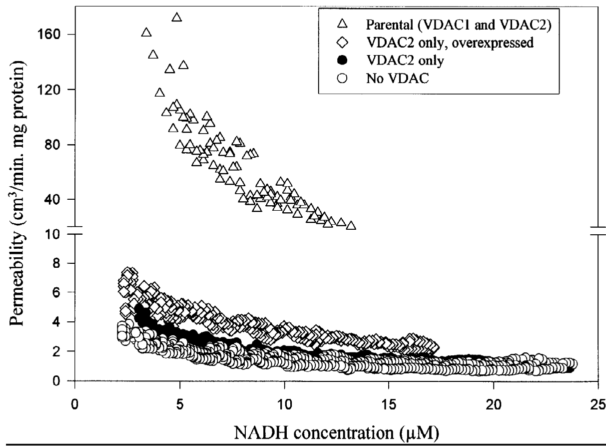

Fig. 4. The comparison of the permeability of the outer membrane of mitochondria from parental yeast (triangles), *V1PV2* yeast (diamonds), Δ*por1* yeast (closed circle) and Δ*por1* Δ*por2* yeast (open circle). The results from three different batches of mitochondria are illustrated for parental and Δ*por1,* two different batches for *V1PV2,* and four different batches for Δ*por1* Δ*por2.*

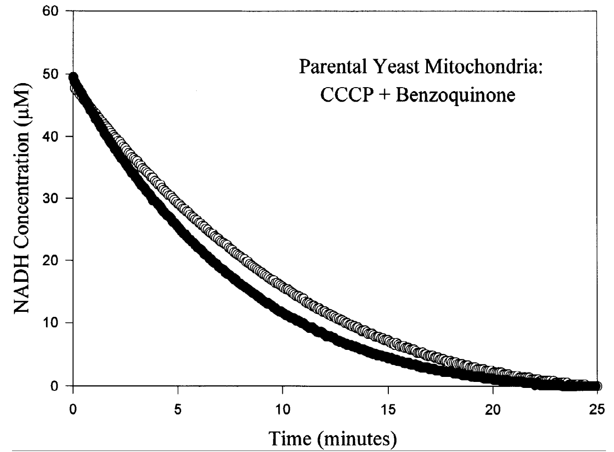

Fig. 5. NADH oxidation by parental cell mitochondria in the presence of the uncoupler, CCCP, and the soluble electron acceptor, benzoquinone. Experiments were done as in Fig. 2 except that 3 μM CCCP (final) and 300 μM benzoquinone (final) was mixed into the mitochondria before the addition of NADH. The data for intact and hypotonically shocked mitochondria are indicated by open and closed circles, respectively. Benzoquinone can react directly with NADH at low rates and the results were corrected for this activity.

Δ*por2,* 0.8 to 3 cm^3/min · mg protein but half that in the cells overexpressing YVDAC2, 2 to 8 cm^3/min · mg protein (Fig. 4).

The Role of Uncoupling in the Permeability Measurements

Uncoupling could account for some of the difference in the rate of NADH oxidation between intact and disrupted mitochondria. The hypotonic treatment used to break the outer membrane could have partly damaged the inner membrane even though there was no significant fumarase leakage. A partial uncoupling could reduce the membrane potential and thus increase NADH oxidation. To control for this, experiments were performed in the presence of uncouplers.

The addition of uncouplers to intact, parental yeast resulted in doubling or tripling the rate of oxidation. Similar increases were observed with the shocked mitochondria. Despite the use of low salt conditions ([KCl] and [$MgCl_2$] reduced from 5 to 2 mM; removed phosphate; added sucrose to maintain osmolarity), there were signs that some of the dehydrogenase was leaching off the mitochondrial inner membrane. Thus an artificial electron acceptor, benzoquinone, was used. Figure 5 shows results in the presence of the uncoupler, CCCP. The small increase in the rate of NADH oxidation for mitochondria from parental cells is still evident in uncoupled mitochondria. Thus, the conclusion that damage of the outer membrane makes NADH more accessible to the dehydrogenase in the intermembrane space, stands.

Unexpectedly, uncouplers had strong inhibitory effects on intact mitochondria from Δ*por1* Δ*por2* cells. We used a variety of fundamentally different uncouplers: CCCP, a lipophilic weak acid that acts as a proton carrier; gramicidin, a cation-selective channel former; and a combination of valinomycin and nigericin, a K^+ carrier with a K^+—H^+ exchanger. The commonality of the inhibition is difficult to understand on the basis of known uncoupler inhibition effects (Heytler, 1963; Kaback et al., 1974) and seems to be rooted in their common property, the increase in H^+ permeability. In view of this, it is unlikely that any small uncoupling resulting from the mild hypotonic shock was responsible for the accelerated NADH oxidation. Nevertheless, we found conditions that resulted in the stimulation of NADH oxidation. Low levels (50 ng/ml) of gramicidin resulted in a small stimulation. The large acceleration of NADH oxidation resulting from the hypotonic shock was evident in the presence of the gramicidin (Fig. 6) even under these conditions.

While the action of uncouplers is complex, the same effect of breaking the outer membrane is seen with the uncouplers present. Thus in both the parental and Δ*por1* Δ*por2* mitochondria, breakage of the outer membrane increases the access of NADH to the dehydrogenase.

The Possible Roles of YVDAC1 and YVDAC2 in Mitochondrial Function

Our results are consistent with the contention that YVDAC1 provides the major pathway for permeability through the mitochondrial outer membrane, since outer membrane permeability to NADH was reduced by a factor of 20 in the absence of YVDAC1. Also supporting

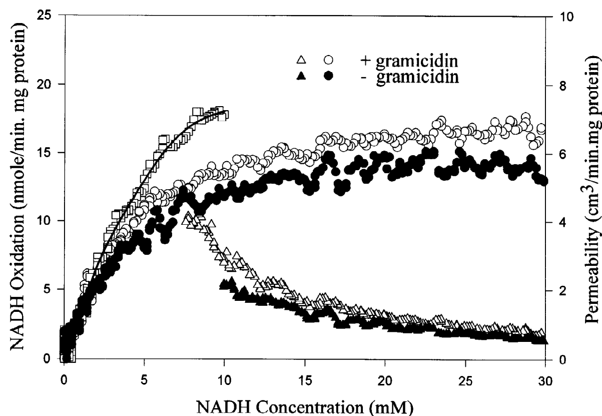

Fig. 6. NADH oxidation by Δ*por1* Δ*por2* yeast mitochondria, in the absence and presence of gramicidin. The data were obtained as described in Fig. 2 and Fig. 3. The oxidation rates by intact (circles) and broken (squares) mitochondria were recorded in the absence (open symbols) and presence (closed symbols) of 50 ng/ml gramicidin (final). Each assay used 107 μg of mitochondrial protein.

this position are the ability of YVDAC1 to form channels in phospholipid membranes (Colombini, 1979; Colombini, Blachly-Dyson & Forte, 1996), and reports that treatments that close reconstituted VDAC channels also inhibit mitochondrial function (*see* Introduction). However, even when YVDAC1 is missing, the outer membrane does have a finite permeability as indicated by a low level of NADH oxidation in intact mitochondria from mutant cells. This residual permeability is not due to the presence of some damaged mitochondria because it is trypsin resistant (the activity of broken mitochondria is trypsin sensitive). Furthermore, it does not appear to be solely due to the presence of YVDAC2, since most of this residual permeability is found in mitochondria missing both VDAC proteins. However, overexpressing YVDAC2 doubles this permeability and thus YVDAC2 may play some role.

Despite the relatively low level of sequence conservation (around 50% identity) between YVDAC1 and YVDAC2, the overall pattern of alternating hydrophilic and hydrophobic residues is conserved (Forte et al., 1996). Based on this beta pattern, YVDAC2 is expected to form a channel. VDAC proteins from humans, which have much lower levels of sequence identity to yeast (~30%), but a similar beta pattern, form channels that are indistinguishable from YVDAC1 channels when introduced into bilayers and can substitute for YVDAC1 in yeast (Blachly-Dyson et al., 1993). Yet, to date, all attempts to reconstitute channel-forming activity from YVDAC2 have failed (Blachly-Dyson et al., 1997).

The small contribution of YVDAC2 to NADH permeability could be explained if YVDAC2 were to form channels that were smaller and less favorable to anions than those formed by YVDAC1. If the M.W. cutoff of YVDAC2 were less than 700 (rather than 5000) it would exclude NADH. The selectivity change would explain why mitochondria isolated from Δ*por1* yeast were observed (Michejda et al., 1990) to require higher (4 mM) Mg^{++} to achieve reasonable ADP-dependent respiration (Mg^{++}-nucleotide complexes are less negative) and were 10–15 times less sensitive to carboxyatractyloside (highly negatively charged) than parental mitochondria (Michejda et al., 1990). In the presence of the 1 mM free Mg^{++} found in the cytosol (Veloso et al., 1973), there could be sufficient flux of ATP, ADP and smaller metabolites through this hypothetical YVDAC2 channel to allow reasonable growth rates. Indeed, the rate of respiration of Δ*por1* cells is the same as that of parental cells if ethanol (which is freely permeant through membranes) is the substrate (Michejda et al., 1990). However, the residual permeability in Δ*por1* Δ*por2* mitochondria might be caused by another outer membrane channel such as the peptide-sensitive channel of the mitochondrial outer membrane, which forms slightly cation-selective channels smaller than VDAC, and is found in mitochondria of parental and Δ*por1* yeast (Fevre et al., 1990). It is also possible that membranous connections between the outer membrane and other cellular organelles may serve as conduits for metabolites.

What is the function of YVDAC2? Yeast cells missing YVDAC1 can grow on glycerol-based media at 30°C, but can not grow in the same media at 37°C. However, these cells can grow on glycerol-based media at 37°C if the YVDAC2 gene is overexpressed (Forte et al., 1996; Blachly-Dyson et al., 1997). At 30°C, cells missing YVDAC1 grow twice as fast if normal levels of YVDAC2 are present. Clearly YVDAC2 can contribute to cell function but its role may not be to form a permeability pathway across the outer membrane. VDAC is known to be the binding site for cytosolic kinases (hexokinase and glycerol kinase), which bind reversibly to the mitochondrial outer membrane, presumably to give them preferential access to ATP generated in the mitochondria (reviewed by Adams et al., 1991). VDAC may also be involved in the association of mitochondria with the cytoskeleton (Linden & Karlsson, 1996). Perhaps YVDAC2 has lost the ability to form channels and instead has specialized in binding to mitochondria-associated proteins. If this is the case, the existence of the two yeast VDAC genes should assist in dissecting the multiple functions of VDAC proteins.

This work was supported by grant # GM 35759 from the National Institutes of Health. AL was the recipient of a fellowship from the Ministry of Education of Taiwan, Republic of China.

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