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NADH Is Specifically Channeled Through the Mitochondrial Porin Channel in *Saccharomyces cerevisiae*

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In many kinds of permeabilized cells, the restriction of metabolite diffusion by a mitochondrial porin "closed state" has been shown to control the respiration rate. However, since in isolated mitochondria the porin appears to be always "open," the physiological relevance of a putative regulation via this channel status is now a subject of discussion. In *Saccharomyces cerevisiae*, in which some of the NADH dehydrogenase active sites are facing the intermembrane space, this regulatory mechanism might play an important role for the regulation of the cytosolic redox status. Using permeabilized spheroplasts from wild-type and porin-deficient mutant, we show that the NADH produced in the cytosol is channeled to the mitochondrial NADH dehydrogenases through a metabolic network involving the porin channel. Thus, the control exerted by the porin (i.e., "open" or "closed" state) seems to be determined through its participation or not in organized metabolic networks.

KEY WORDS: NADH; mitochondrial porin channel; permeability; metabolic regulation; channeling; yeast; porin-deficient mutant; oxidative phosphorylation.

INTRODUCTION

In most nonphotosynthetic and aerobic cells, mitochondrial oxidative phosphorylation is the main process for ATP synthesis. Both ATP utilization and substrate delivery to the respiratory chain participate in the regulation of oxidative phosphorylation (for reviews, see Balaban, 1990; Brown, 1992). From numerous studies on mammalian and yeast mitochondria (Beauvoit *et al.*, 1989; Doussière *et al.*, 1984; Gellerich *et al.*, 1983; Groen *et al.*, 1982; Mazat *et al.*, 1986; Rigoulet, 1990; Rigoulet *et al.*, 1987; Tager *et al.*, 1983), it was established that the control of oxidative phosphorylation is shared by different steps and is dependent on external and internal conditions. We previously showed that the oxidative phosphorylation yield (ATP/O) depends on the rate of the flux through the proton pumps. The lower the flux through the respiratory chain, the higher the ATP/O ratio (Avéret *et al.*, 1998; Fitton *et al.*, 1994; Ouhabi *et al.*, 1989). The flux may be controlled upstream the respiratory chain, at the level of substrate supply or already at the level of cytosolic or mitochondrial NADH production catalyzed by cytosolic or matrix dehydrogenases, respectively (Ouhabi *et al.*, 1989; Rigoulet *et al.*, 1985). Yeast mitochondria are able to oxidize cytosolic NADH because they possess NADH dehydrogenases (the so-called external NADH dehydrogenases) located towards the outer surface of the inner membrane (von Jagow and Klingenberg, 1970). Cytosolic NADH has to cross the mitochondrial outer membrane to deliver electrons to the external dehydrogenases. However, the diffusion of metabolites through the porin channel has never been suggested as a major controlling step for oxidative phosphorylation in isolated mitochondria.

Several studies on permeabilized heart and liver cells have shown that the apparent $K_{0.5}$ for ADP versus the rate of respiration is higher than that measured in mitochondria isolated from the same tissues. Osmotic disruption of the outer mitochondrial membrane in fibers leads to a $K_{0.5}$

Key to abbreviations: Tris, 2-amino-2-hydroxymethyl-1,3-propandiol; EGTA, ethylene glycol-bis(β-aminoethylether)-N, N, N, N'-tetreacetic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

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for ADP close to that observed in isolated mitochondria (Fontaine *et al.*, 1995; Saks *et al.*, 1991, 1993, 1995a,b). This fact has been interpreted as a proof that metabolite diffusion through the porin channel is restricted in permeabilized heart and liver cells. Thus, an important site of respiratory control in vivo was suggested to be the porin channel of the outer mitochondrial membrane. We showed a retarded diffusion for ADP also in permeabilized yeast, similar to the one observed in permeabilized mammalian cells (Avéret *et al.*, 1998). This is true for several respiratory substrates; the $K_{0.5}$ for each substrate versus the respiration rate in permeabilized spheroplasts always exceeds the one observed on isolated mitochondria. In addition, in a porin-deficient mutant, $K_{0.5}$ for NADH is not significantly different in isolated mitochondria and permeabilized spheroplasts and is comparable to $K_{0.5}$ measured in wild-type permeabilized spheroplasts (Avéret *et al.*, 1998). Therefore, the retarded diffusion seems essentially because of the presence of the porin channel in a closed state. However, from a physiological point of view it is difficult to unanimously explain these two observations, since in isolated mitochondria the porin channel seems to be always "open," while in permeabilized spheroplasts it is always "closed" (Avéret *et al.*, 1998). Consequently, further investigations enlightening the regulatory mechanisms behind the "opening–closing" of the porin are necessary. Intriguingly, the cellular NADH concentration measured in vivo is about 0.2 mM (Anderlund *et al.*, 1999), i.e., in between $K_{0.5}$ for NADH as respiratory substrate measured in isolated mitochondria and the one measured in permeabilized spheroplasts. We propose that the porin physiological status ("open" or "closed") may be, in some conditions, a key determinant of the reoxidation rate of cytosolic NADH by the respiratory chain.

In this study, we observed that $K_{0.5}$ for NADH in permeabilized spheroplasts was similar to the one measured in isolated mitochondria, only when the substrate NADH was produced by endogenous cytosolic NAD^+-dependent dehydrogenases (i.e., glyceraldehyde-3-phosphate dehydrogenase or cytosolic alcohol dehydrogenase). Data obtained using a porin-deficient mutant confirm an "open" state of the porin when NAD^+-dependent dehydrogenases are working in permeabilized spheroplasts from the wild type. $K_{0.5}$ for added NADH was not improved (lowered) as a result of the activity of other enzymes, which are not involved in NADH production (e.g., hexokinase and $NADP^+$-dependent glucose-6-phosphate dehydrogenase). Moreover, the activation of NAD^+-dependent dehydrogenases did not affect the restricted diffusion of ADP through the outer membrane showing that the functioning of these enzymes did not simply "open" the porin channel for any metabolite. In conclusion, our data strongly indicated that the NADH produced in the cytosol by dehydrogenases is channeled to the respiratory chain through a structured metabolic pathway including the porin channel.

MATERIALS AND METHODS

Yeast Aerobic Cultures

The strains used were maintained on YPD plates. The diploid wild-type strain of *Saccharomyces cerevisiae* (yeast foam) was grown in an incubator (New Brunswick) at 28°C in complete medium: 1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, supplemented with 2% lactate as carbon source. The haploid wild-type strain of *S. cerevisiae* DBY747 (*MAT a, ura3–52, leu2–3, leu2–112, his3Δ1, trp1–289a*) and porin-deficient mutant *S. cerevisiae* DBY747/B5 (*MAT a, Δpor-:: URA3, ura3–52, leu2–3, leu2–112, his3Δ1, trp1–289a*) (Dihanich *et al.*, 1987; Guo and Lauquin, 1996; Michejda *et al.*, 1989) were grown in the same medium and under the same conditions. Growth was followed by optical density measurements (600 nm), and the cells were harvested in the logarithmic growth phase.

Preparation and Permeabilization of Spheroplasts

Cells were washed twice with 20-mL distilled water and incubated for 10 min (yeast foam) or 30 min (porin-deficient mutant and haploid wild-type strain) on a rotary shaker at 32°C in 0.5 M β-mercaptoethanol and 0.1 M Tris-HCl (pH 9.3). Subsequently, cells were washed three times with 0.5 M KCl, 10 mM Tris-HCl (pH 7), and thereafter (1-g dry weight of cells) suspended in 10 mL of 1.35 M sorbitol, 1 mM EGTA, 10 mM citrate-phosphate (pH 5.8). To the yeast foam or to the porin-deficient mutant and haploid wild-type strains, 0.17- or 1-g cytohelicase, respectively, was added and the cells were then incubated for about 50 min at 32°C with gentle shaking. The obtained spheroplasts were centrifuged at 800*g* for 5 min, washed three times with the same solution (1.35 M sorbitol, 1 mM EGTA, 10 mM citrate-phosphate (pH 5.8)), and thereafter suspended in the following buffer (Medium 1): 1 M sorbitol, 0.5 mM EGTA, 2 mM $MgSO_4$, 1.7 mM NaCl, 10 mM NH_4Cl, 10 mM potassium phosphate (pH 6.8), 0.1% bovine serum albumin.

The protein concentration was estimated by the biuret method using bovine serum albumin as a standard.

Permeabilized spheroplasts were obtained after incubation at a concentration corresponding to 1 mg protein per mL in Medium 1 for 10 min at 28°C with 20 $\mu g \cdot mL^{-1}$ nystatin (Avéret *et al.*, 1998).

Preparation of Isolated mitochondria

Mitochondria were isolated from intact spheroplasts as previously described by Guérin *et al.*(1979).

Respiration Measurement

Oxygen consumption rates were measured at 28°C with a Clark oxygen electrode (Gilson) in a 2-mL thermostatically controlled chamber. Spheroplasts and mitochondria were incubated in Medium 1.

In experiments involving cytosolic NAD^+-dependent dehydrogenases (glyceraldehyde-3-phosphate dehydrogenase or alcohol dehydrogenase), various NAD^+ concentrations were added to permeabilized spheroplasts in the presence of the corresponding substrate (fructose-1,6-bisphosphate or ethanol). Activation of glyceraldehyde-3-phosphate dehydrogenase was induced by addition of ADP (2 mM) and fructose-1-6-bisphosphate (10 mM) rather than glyceraldehyde-3-phosphate due to its instability. When the respiration rate reached steady state, samples were taken and precipitated by methanol (90%)/KOH (2 N) mixture at 55°C. The extract was subsequently analyzed for the amount of NADH formed.

Determination of NADH Concentration in Methanolic KOH Extracts

NADH content in neutralized methanolic KOH extracts was fluorimetrically measured after addition of dihydroxyacetone-phosphate and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) as previously described by Klingenberg (1985) using a Kontron fluorimeter (excitation wavelength was 340 nm and fluorescence emission wavelength was 460 nm).

Data Analysis

From plots of the initial rate (JO) versus substrate concentration ([NADH] or [ADP]), we determined the value of V_m and $K_{0.5}$ by fitting the data to the Michaelis–Menten equation using the nonlinear squares fit. It is well known that this treatment applies to numerous mechanisms more complex than the Michaelis–Menten mechanism and in which V_m and $K_{0.5}$ are defined in a more complex way. This is particularly true when considering a metabolic pathway in which the kinetic control distribution may vary as a function of the flux. However, when experimental data fit the following general equation, $v = V_m[S]/(K_{0.5} + [S])$, the curve is a rectangular hyperbola whose asymptotes are $S = -K_{0.5}$ and $v = V_m$. Thus, for very low values of respiratory substrate concentrations (very small compared to $K_{0.5}$), the initial rate is directly proportional to $[S]$ and $v = V_m[S]/K_{0.5}$. During such conditions, $V_m/K_{0.5}$ corresponds to a rate constant for the reaction (Cornish-Bowden, 1976). Moreover, in isolated yeast mitochondria, it has been shown that for very small values of respiratory substrate concentrations, the flux control coefficient of the NADH dehydrogenase over respiratory rate is very high (near 1) (Pahlman *et al.*, 2002) and we can assume that the $V_m/K_{0.5}$ ratio is, for such a complex mechanism, the most easy and accurate kinetic parameter of this dehydrogenase. By analogy with a well-defined enzyme parameter, i.e., k_{cat}/K_m, we called $V_m/K_{0.5}$ enzyme efficiency.

RESULTS AND DISCUSSION

The Efficiency of the Respiratory Chain Towards Externally Added NADH Is Larger in Isolated Yeast Mitochondria Than in Permeabilized Spheroplasts

We measured respiration rates of both isolated mitochondria and permeabilized spheroplasts at steady state in the presence of a saturating ADP concentration and various concentrations of externally added NADH. Kinetic parameters (V_m and $K_{0.5}$ for NADH) were obtained by direct fitting of the data using the nonlinear least squares fit as shown in Fig. 1.

Table I shows the kinetic parameters of NADH-linked respiration determined under both phosphorylating and uncoupled conditions (with CCCP) in isolated mitochondria and permeabilized spheroplasts. The values of V_m did not differ significantly in isolated mitochondria and in permeabilized spheroplasts, while $K_{0.5}$ for NADH was about 30 times lower in isolated mitochondria. Compared to phosphorylating conditions, uncoupling with CCCP increased V_m and $K_{0.5}$ for NADH in both isolated mitochondria and spheroplasts to approximately the same extent. Consequently, the efficiency, defined as the ratio between V_m and $K_{0.5}$ was not affected by addition of the uncoupler CCCP.

The fact that $K_{0.5}$ for NADH was so much lower in isolated mitochondria than in permeabilized spheroplasts indicated the presence of a controlling step upstream the NADH dehydrogenase in permeabilized spheroplasts. Because we have previously established that the plasma membrane barrier is not limiting small molecules diffusion in permeabilized spheroplasts (Avéret *et al.*, 1998), the main controlling step in the respiration was not the NADH

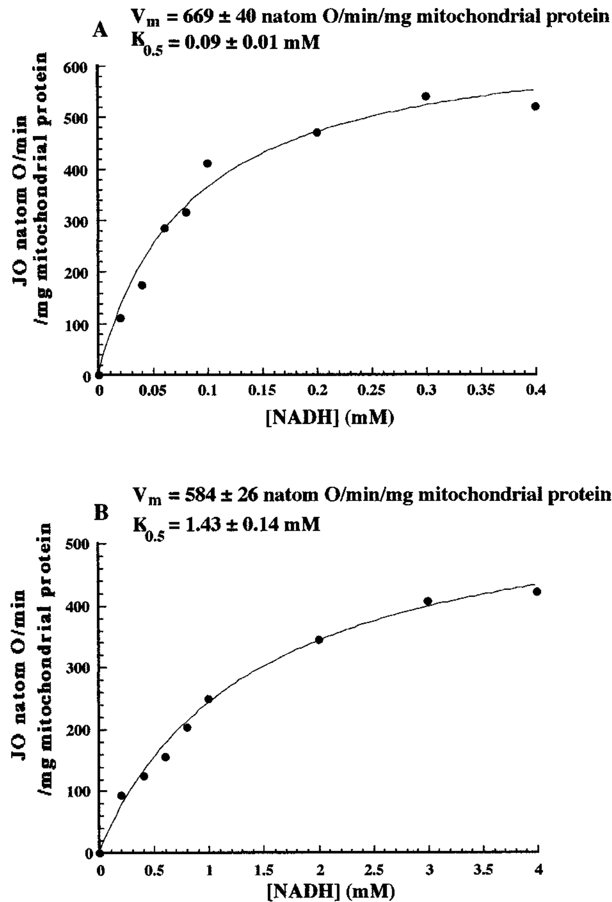

Fig. 1. Dependence of the phosphorylating respiration on exogenous NADH concentration in yeast isolated mitochondria (A) and permeabilized spheroplasts (B). Respiration fluxes were measured as described under Materials and Methods in medium 1 containing either 1 or 2 mM ADP for mitochondria and spheroplasts, respectively, and various concentrations of NADH. Spheroplasts (1 mg protein per mL) were permeabilized with 20 $\mu g \cdot mL^{-1}$ nystatin. Respiration rates were expressed as natom $O \cdot min^{-1} \cdot mg^{-1}$ mitochondrial protein assuming that 1-mg spheroplast protein contains 0.32-mg mitochondria protein (Avéret *et al.*, 1998). The V_m and $K_{0.5}$ values were calculated from the nonlinear squares fit of one typical experiment; similar results were obtained from two other preparations.

dehydrogenase itself, but the diffusion of NADH through the cytosol and/or the outer mitochondrial membrane.

The Efficiency of the Respiratory Chain Was High When NADH Was Produced by Cytosolic NAD^+-Dependent Dehydrogenases in Permeabilized Spheroplasts

To bypass a potential diffusion limitation of NADH through the cytosol of permeabilized spheroplasts, NADH was generated by cytosolic NAD^+-dependent dehydrogenases (such as the glyceraldehyde-3-phosphate dehydrogenase or the cytosolic alcohol dehydrogenase) instead of externally supplied (Table II). The efficiency towards NADH produced by these enzymes increased up to a value close to the level of the efficiency towards externally supplied NADH of isolated mitochondria (Table I). To ensure that this result was not simply an effect of NAD^+ being added as a cofactor, we studied the effect of supplying permeabilized spheroplasts with different external concentrations of NAD^+ and NADH resulting in various NAD^+/NADH ratios (values of either 0.2, 5, or 20 were kept constant during each experiment) on kinetic parameters. In all cases, respiratory efficiencies were low (307, 342, and 210 natom O/min/mg protein per mM, respectively) demonstrating that the high efficiencies (Table II) resulted from cytosolic production of NADH rather than from an altered redox state of the permeabilized cells.

Porin Deficiency Increase $K_{0.5}$ of the Respiration Rate for NADH When Produced in the Cytosol of Permeabilized Spheroplasts

Numerous studies showed that, in mitochondria isolated from yeast missing the gene encoding the POR1, the permeability to NADH was largely decreased indicating that porin1 is the major pathway for NADH flux to the external NADH dehydrogenases (Avéret *et al.*, 1998; Lee *et al.*, 1998; Michejda *et al.*, 1994). In view of these previous works, we reasoned that the increase in efficiencies corresponds to an "open state" situation of the porin channel. On the basis of this assumption, a strain deleted of the porin would not show any difference in efficiency towards externally added and cytosolically produced NADH. Figure 2 shows the respiration rate dependency on NADH measured after activation of the glyceraldehyde-3-phosphate dehydrogenase in permeabilized spheroplasts prepared from both a wild-type strain and a mutant deficient in the gene encoding the porin. Clearly, for the same concentration of NADH formed at steady state, the respiration rate of the mutant was very low compared to wild type and the lack of porin led to a large rise of $K_{0.5}$ for NADH. This experiment shows the direct involvement of porin in the process controlling the permeability of the cytosolic produced NADH through the outer mitochondrial membrane. It is worth noticing that, in the mutant, the experimental conditions, i.e., production of NADH by glyceraldehyde-3-phosphate dehydrogenase from NAD^+, did not allow us to reach as high NADH concentration at steady state as in the wild type. Therefore, we could not determine the kinetic parameters in this case.

Table I. Kinetic Parameters of NADH-Linked Respiration in Isolated Yeast Mitochondria and in Permeabilized Spheroplasts

Condition	V_m (natom O · min^{-1}. mg^{-1} mitochondrial protein)	$K_{0.5}$ for NADH (mM)	Efficiency (natom O · min^{-1} · mg^{-1} mitochondrial protein per mM)
Isolated mitochondria			
+ADP + various [NADH]	542 ± 152	0.06 ± 0.03	9072 ± 1275
+CCCP + various [NADH]	866 ± 257	0.12 ± 0.03	7138 ± 875
Permeabilized spheroplasts			
+ ADP + various [NADH]	638 ± 25	1.56 ± 0.17	386 ± 104
+ CCCP + various [NADH]	1041 ± 200	3.93 ± 1.95	244 ± 73

Note. Spheroplasts and mitochondria were prepared as described under Materials and Methods. Respiration rates were measured in Medium 1 as described under Materials and Methods with either 0.5 mg protein per mL of mitochondria or spheroplasts corresponding to 1 mg protein per mL (spheroplasts were permeabilized by 20 μg · mL^{-1} nystatin) and expressed as natom O · min^{-1} · mg^{-1} mitochondrial protein. Kinetic parameters were measured in the phosphorylating state (including either 1 mM ADP or 2 mM ADP for mitochondria and spheroplasts, respectively) or in uncoupling state (+10 μM CCCP) after the addition of various concentrations of NADH to either isolated mitochondria or permeabilized spheroplasts. V_m and $K_{0.5}$ values were calculated as the mean from at least three determinations ±SD. Efficiency was calculated for each experiment and expressed as the mean ±SD.

The Efficiency of the Respiratory Chain Was Not Improved by Other Enzyme Activities When NADH Was Supplied Externally to Permeabilized Spheroplasts

Next we wanted to exclude that any other cytosolic enzyme activities could increase the respiratory efficiency towards externally added NADH. We therefore added NADH externally when the $NADP^+$-dependent glucose-6-phosphate dehydrogenase or hexokinase was active. The obtained efficiencies were in both cases about 600 natom O/min/mg protein per mM (Table III). Consequently, when we activated enzymes that were not NAD^+-dependent dehydrogenases, the respiratory efficiency with NADH as externally added substrate was similar to that obtained in permeabilized spheroplasts in the phosphorylating state (Table I). Thus, an increase in efficiency seems to occur only when NADH is produced by cytosolic dehydrogenases as compared to supplied in the external medium. Altogether, our results so far support the idea that NAD^+-dependent dehydrogenases promote the "opening" of the porin channel, which in turn allows better diffusion of NADH to the respiratory chain. Consequently, when the porin is "closed," we measure a lower efficiency of the respiratory chain towards NADH because the local concentration of NADH near the external NADH

Table II. Kinetic Parameters of NADH-Linked Respiration in Permeabilized Spheroplasts When Cytosolic NAD^+-Dependent Dehydrogenases Produced NADH in the Cytosol

Condition	V_m (natom O · min^{-1}. mg^{-1} mitochondrial protein)	$K_{0.5}$ for NADH (mM)	Efficiency (natom O · min^{-1} · mg^{-1} mitochondrial protein · mM^{-1})
+Fructose 1,6-diphosphate			
+ADP + various [NAD^+]	426 ± 127	0.07 ± 0.02	6970 ± 1883
+Ethanol + ADP + various [NAD^+]	219 ± 23	0.08 ± 0.05	3509 ± 1331
+Ethanol + CCCP + various [NAD^+]	445 ± 86	0.09 ± 0.01	4967 ± 573

Note. Respiration rates were measured in Medium 1 as described under Materials and Methods with spheroplasts corresponding to 1 mg of spheroplasts protein per mL, permeabilized with 20 μg · mL^{-1} nystatin, and expressed as natom O · min^{-1} · mg^{-1} mitochondrial protein. Kinetic parameters were determined when either glyceraldehyde-3-phosphate dehydrogenase (the used substrate was fructose-1-6-bisphosphate (10 mM), rather than glyceraldehyde-3-phosphate due to its instability and ADP (2 mM) or alcohol dehydrogenase produced NADH during the state of phosphorylation (the used substrate was ethanol (100 mM) and ADP (2 mM) and uncoupled state (use of ethanol (100 mM) and CCCP (10 μM). Ethanol addition first stimulated mitochondrial alcohol dehydrogenase and NAD^+ addition stimulated cytosolic alcohol dehydrogenase. Consequently, the strictly dependent respiration rate on external NADH dehydrogenase activation was the difference between the respiratory rate obtained after and before NAD^+ addition. During all conditions, at each steady after addition of NAD^+, samples were taken and extracted in methanolic KOH at 55°C, in order to determine the concentration of NADH formed as described under Materials and Methods. V_m and $K_{0.5}$ values were calculated as the mean of at least three determinations ±SD. Efficiency was calculated for each experiment and expressed as the mean ±SD.

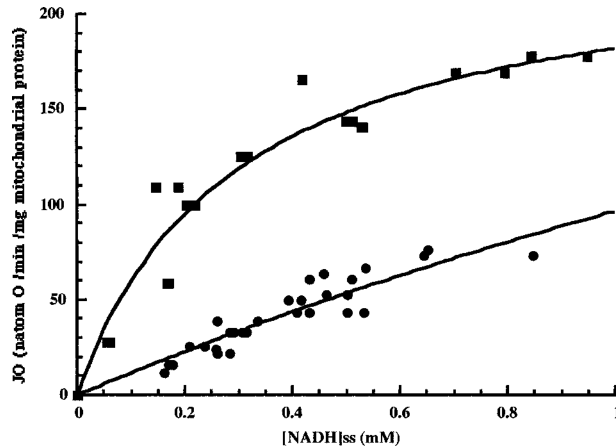

Fig. 2. Effect of porin deficiency on the dependence of respiration on NADH concentration produced by glyceraldehyde-3-phosphate dehydrogenase activity in permeabilized spheroplasts. Spheroplasts were isolated as described under Materials and Methods from either a porin-deficient mutant (●) or the parent strain (■). Respiration fluxes were measured as described under Materials and Methods in Medium 1 containing 1-mg spheroplast protein per mL permeabilized with 20 $\mu g \cdot m1^{-1}$ nystatin, fructose-1-6-bisphosphate (10 mM), ADP (2 mM), and various concentrations of NAD^+. At each steady state reached after addition of NAD^+, samples were taken to determine the concentration of NADH formed ($[NADH]_{55}$) as described under Materials and Methods. The same comparison was done three times on different spheroplast preparations.

dehydrogenases is lower than the global NADH content of the cell (which is the only parameter that we can assess experimentally). In contrast, when the porin is "open," the measured NADH concentration is virtually similar to the intermembrane space concentration since diffusion is no longer limited. This point is important, as our data do not support an intrinsic change in the response of the respiratory chain towards NADH.

The Efficiency of the Respiratory Chain Towards ADP Was Not Affected When NADH Was Produced by Cytosolic Alcohol Dehydrogenases in Permeabilized Spheroplasts

To generalize our observation, we examined the efficiency of the respiratory chain towards ADP. As it has been previously shown that β-NADH decreases the permeability of the mitochondrial outer membrane to ADP while α-NADH does not (Lee *et al.*, 1994), we used cytosolic alcohol dehydrogenase well known to produce α-NADH. We found that the respiratory chain $K_{0.5}$ for ADP was lower in isolated mitochondria than in permeabilized spheroplast (Table IV) (see also Avéret *et al.*, 1998). It therefore seems like a decreased efficiency with different externally added substrates is a general feature of permeabilized spheroplasts compared to isolated mitochondria. To further ascertain that the increased efficiency when NADH was produced in the cytoplasm was linked to the opening of the porin channel, we supplied different concentrations of ADP while the delivery of NADH to the respiratory chain was ensured by the cytosolic alcohol dehydrogenase (conditions during which we postulated that the porin "opened"). Our prediction was that the "opening" of the porin would increase the efficiency of the respiratory chain towards ADP, as it would be able to diffuse through the "open" porin channel. This did not happen and no increase of the respiratory efficiency towards ADP was observed. Consequently, this indicates that the obtained increase in efficiency when NADH is produced by endogenous dehydrogenases is porin-dependent but the porin is a channel discriminating different substrates.

Taken together, these results demonstrate the direct involvement of the porin in the increase in the oxidative phosphorylation efficiency towards NADH. This suggests that the porin favors NADH permeability through the

Table III. Kinetic Parameters in Permeabilized Spheroplasts When Cytosolic NAD^+-Independent Metabolic Pathways Were Active

Condition	V_m (natom $O \cdot min^{-1}$. mg^{-1} mitochondrial protein)	$K_{0.5}$ for NADH (mM)	Efficiency (natom $O \cdot min^{-1} \cdot mg^{-1}$ mitochondrial protein $\cdot mM^{-1}$)
+ Glucose-6-phosphate + $NADP^+$ + ADP + various [NADH]	773 ± 105	1.54 ± 0.73	600 ± 213
+ Glucose + ATP + various [NADH]	718 ± 102	1.53 ± 0.4	522 ± 203

Note. Respiration rates were measured in Medium 1 as described under Materials and Methods with spheroplasts corresponding to 1 mg of spheroplasts protein per mL, permeabilized with 20 $\mu g \cdot mL^{-1}$ nystatin, and expressed as natom $O \cdot min^{-1} \cdot mg^{-1}$ mitochondrial protein. Kinetic parameters were determined when $NADP^+$-dependent glucose-6-phosphate dehydrogenase was active; various concentrations of NADH were added 2 min after the addition of glucose-6-phosphate (10 mM), $NADP^+$ (1 mM), and ADP (2 mM). During the second condition, kinetic parameters were measured when hexokinase was active; various concentrations of NADH were added 2 min after addition of glucose (10 mM) and ATP (1 mM). V_m and $K_{0.5}$ values were calculated as the mean of at least three determination ±SD. Efficiency was calculated for each experiment and expressed as the mean ±SD.

Table IV. Kinetic Parameters for ADP Versus Respiration Rate on Ethanol in Isolated Yeast Mitochondria and in Permeabilized Spheroplasts

Condition	V_m (natom O · min^{-1}. mg^{-1} mitochondrial protein)	$K_{0.5}$ for ADP (mM)	Efficiency (natom O · min^{-1} · mg^{-1} mitochondrial protein per mM)
Isolated mitochondria			
+ EtOH + various [ADP]	258 ± 24	0.05 ± 0.01	5585 ± 1620
Permeabilized spheroplasts			
+ EtOH + various [ADP]	159 ± 38	0.80 ± 0.34	197 ± 63
+ EtOH + NAD^+ + various [ADP]	256 ± 72	1.21 ± 0.50	186 ± 66

Note. Respiration rates were measured in Medium 1 as described under Materials and Methods with spheroplasts corresponding to 1 mg of spheroplasts protein per mL, permeabilized with 20 μg · mL^{-1} nystatin, and expressed as natom O · min^{-1} · mg^{-1} mitochondrial protein. Kinetic parameters were measured at steady state after addition of various concentrations of ADP to either isolated mitochondria or to permeabilized spheroplasts. The presence of NAD^+ to permeabilized spheroplasts stimulated cytosolic alcohol dehydrogenase. V_m and $K_{0.5}$ values were calculated as the mean of at least three determinations ±SD. Efficiency was calculated for each experiment and expressed as the mean ±SD.

mitochondrial outer membrane. Nevertheless, because ADP diffusion was not increased when NAD^+-dependent cytosolic dehydrogenases were working, we cannot explain our observation by a general "opening–closing" mechanism of the porin allowing unspecific diffusion of any metabolite. We believe that our results strongly support the idea that NADH is channeled through the porin channel, which might be part of a structured metabolic network regulated by the activity of, at least, some cytosolic dehydrogenases. This channeling mechanism would provide the NADH from cytosolic NAD^+-dependent dehydrogenases to NADH dehydrogenases located on the external side of the inner mitochondrial membrane, in order to be quickly reoxidized. Another channeling process involving cytosolic enzymes, porin, and mitochondrial membrane enzymes is described in the work of Seppet *et al.* (2001), who demonstrated that about 30% of the cellular pool of adenine nucleotides in muscle cells is actively involved in metabolic channeling pathways: ADP is released from the MgATPases of reticulum and myofibrils into the vicinity of mitochondria.

An alternative explanation of our results would be a binding of some cytosolic dehydrogenases, i.e., the glyceraldehyde-3-phosphate dehydrogenase and the alcohol dehydrogenase, in the vicinity of the porin channel. In this case, the NADH generated by dehydrogenases linked to the mitochondrial outer membrane is directed towards the two external NADH dehydrogenases. Such a metabolic organization (i.e., mitochondrially bound hexokinase producing ADP effectively recycled back to oxidative phosphorylation apparatus) has been shown in rat brain (BeltrandelRio and Wilson, 1991; Inui and Ishibashi, 1979; Moore and Jobsis, 1970; Wilson, 1984; Wilson and Felgner, 1977).

In *S. cerevisiae*, Grandier-Vazeille *et al.* (2001) have recently shown the existence of a supramolecular complex, using colorless native polyacrylamide gel electrophoresis (CN-PAGE). This complex linked the two external NADH dehydrogenases, glycerol-3-phosphate dehydrogenase and D- and L-lactate dehydrogenases (i.e., five intermembrane space-facing dehydrogenases), with the matrix facing NADH-dehydrogenase, several tricarboxylic acid cycle, and matrix enzymes. In addition, these authors systematically identified a high-molecular-weight complex of several cytosolic proteins, which they called "contaminating proteins," within which isoforms I and III of glyceraldehyde-3-phosphate. Porin was not detected in this work but we here perform functional analyses that clearly indicate that the channeling network for NADH from cytosolic dehydrogenases to the respiratory chain involves the porin channel. To determine which other enzymes are involved in this process, more functional studies of different mutants are needed.

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