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# The access of metabolites into yeast mitochondria in the presence and absence of the voltage dependent anion selective channel (YVDAC1)

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**Since yeast *Saccharomyces cerevisiae* mutants depleted of the voltage dependent anion selective channel (YVDAC1) are still able to grow on a non-fermentable carbon source, a functional transport system in the outer mitochondrial membrane must exist to support the access of metabolites into mitochondria. It was assumed that the properties of the system could be inferred from the differences in the results observed between wild type and mutant mitochondria since no crucial differences in this respect between the two types of mitoplasts were observed. YVDAC1-depleted mitochondria displayed a highly reduced permeability of the outer membrane, which was reflected in increased values of $K_{0.5}^{NADH}$ for respiration and $K_{0.5}^{ADP}$ for triggering phosphorylating state as well as in delayed action of carboxyatractylate (CATR) in inhibition of phosphorylating state. The parameters were chosen to express the accessibility of the applied species to the intermembrane space. The passage of the molecules through the outer membrane depleted of YVDAC1 could be partially improved in the presence of bivalent cations ($Mg^{2+}$, $Ca^{2+}$), as in their presence lower values of the calculated parameters were obtained. The restrictions imposed on the transport of molecules through the YVDAC1-depleted outer membrane resulted in a competition between them for the access to the intermembrane space as measured by changes in parameters observed for a given species in the presence of another one. The competition was stronger in the absence of $Mg^{2+}$ and depended on charge and size of transported molecules, as the strongest competitor was CATR and the weakest one – NADH. Thus, it can be concluded that the transport system functioning in the absence of YVDAC1 is modulated by bivalent cations and charge as well as size of transported molecules. Since an increased level of respiration due to the dissipation of $\Delta\psi$ causes**

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Abbreviations: BSA, bovine serum albumin; CATR, carboxyatracylate; DHFR, dihydrofolate reductase; PSC, peptide sensitive channel; TOM, translocase of outer membrane; TPP, tetraphenylphosphonium; YVDAC1, voltage dependent anion selective channel.

**an increase of $K_{0.5}^{NADH}$ in both wild type and YVDAC1-depleted mitochondria it is concluded that a common property of YVDAC1 and the system functioning in YVDAC1-depleted mitochondria seems to be the dependence of the capacity on the level of mitochondrial respiration.**

The energetic and metabolic functions of mitochondria require effective transport of metabolites through both mitochondrial membranes. While the inner membrane contains a system of specific carriers, effectively exchanging molecules between the matrix and the intermembrane space, the outer membrane acts as a non-specific molecular filter allowing the passage of low molecular mass hydrophilic solutes. The permeability properties of the outer membrane are attributed to the voltage-dependent anion-selective channel (VDAC), known also as mitochondrial porin [1, 2]. It is clear now that this protein is not the only one capable of transporting of metabolites through the outer membrane, because several VDAC-depleted yeast mutants have been found to be viable on non-fermentable carbon source [3–8]. Moreover, cells and isolated mitochondria of one of the mutant strains, B5 [3, 8], were capable of oxidative phosphorylation, which indicates an efficient traffic of adenine nucleotides through the outer mitochondrial membrane [5, 6, 9].

Simultaneously, outer membrane proteins extracted from mitochondria of another VDAC-depleted mutant [4] and reconstituted in a planar lipid bilayer revealed the presence of a diffusion pore with a diameter smaller than that of VDAC and of slight cationic selectivity ([10, 11], our unpublished results). The single-channel characteristics of this pore corresponded closely to the requirements established with a bioenergetic approach for the effective access of large size anionic molecules to the intermembrane space of intact mitochondria isolated from mutant B5 [5, 6, 9]. This putative pore of originally unknown function is thought to coexist with VDAC and take over its function in VDAC-depleted mutants [10, 11]. Recently, a new yeast protein (YVDAC2) was identified that, when overexpressed, suppressed the disruption of the gene encoding VDAC (YVDAC1) [7]. However, YVDAC2 displays low permeability for metabolites [12]. Thus, the protein(s) responsible for metabolite transport through the outer membrane depleted of YVDAC1 is still unidentified.

In the present paper a functional approach was used to describe more closely the characteristic features of the transport system supporting the viability of B5 mutant (depleted of YVDAC1) on a non-fermentable carbon source. The capacity of the system is modulated by the presence of bivalent cations ($Mg^{2+}$ and $Ca^{2+}$) and depends on charge and size of transported species as well as on the level of mitochondrial respiration.

## MATERIALS AND METHODS

***Cell culture.*** Wild type strain of *Saccharomyces cerevisiae* DBY747 (*Matα, ura3-52, leu2-3, leu2-112, his3-Δ1, trp1-289*) and the VDAC (YVDAC1)-depleted mutant B5, derived from the above parent strain [5, 8] were the source of mitochondria. Cultures were grown at 28°C on YP medium (1% yeast extract, 2% peptone), at pH 5.5. Glycerol (3%) was used as a non-fermentable carbon source. Growth was monitored by determining the absorbance at 546 nm and the cells were collected in the log phase, at $A_{546}$ of about 1.0. The growth rate of mutant cells was slightly slower than that of the wild type (doubling time 4 and 3 h, respectively).

***Isolation of mitochondria.*** Mitochondria were isolated essentially as described in [13] with a yield of 4–5 mg of mitochondrial protein per 1 g of wet mass cells. Mitoplasts were obtained by the swelling-shrinking procedure according to [13]. 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 $MgCl_2$, 0.2% BSA. The degree of integrity of the outer mitochondrial membrane was monitored by the permeability of the membrane for exogenous cytochrome *c* as in [14]. Mitochondrial protein was measured by the biuret method, using KCN to account for the turbidity due to phospholipids.

***Determination of* $K_{0.5}$ *values.*** $K_{0.5}$ values, representing concentrations of the studied compounds resulting in half-maximum of their activity, were calculated on the basis of maximal rate of oxygen uptake driven by these compounds. Mitochondria respiration was monitored at 25°C with a Rank oxygen electrode in an incubation medium (2.5 ml) containing: 0.65 M mannitol, 10 mM Hepes, pH 6.9, 10 mM $KP_i$, 5 mM KCl, 0.2% BSA (defatted), ± 4 mM $MgCl_2$ and about 1 mg mitochondrial protein. The amount of oxygen in 1 ml of the medium was assumed at 220 nmoles. Ethanol (20 mM) or NADH at various concentrations was used as the respiratory substrate. The uncoupled state was generated by valinomycin at a final concentration of 0.16 $\mu$g per mg of mitochondrial protein. Values of $K_{0.5}^{Mg}$ were obtained at NADH and ADP at 1 mM for wild type and 1.5 mM for mutant mitochondria.

***Determination of CATR access.*** Carboxyatractylate (CATR) accessibility to the inner membrane was expressed as the time required to complete its inhibitory effect on the phosphorylating state, measured by changes of the transmembrane potential ($\Delta\psi$). $\Delta\psi$ was measured according to [15], with a tetraphenylphosphonium ($TPP^+$)-specific electrode inserted into a Rank oxygen electrode vessel, thus simultaneously with oxygen uptake. Values of $\Delta\psi$ (in mV) were calculated as in [15]. The volume of the mitochondrial matrix, measured as the difference in [$^{14}$C]sucrose and [$^3$H]$H_2O$ distribution in mitochondrial compartments, was taken as 1.4 $\mu$l per 1 mg of mitochondrial protein.

***Miscellaneous.*** The concentration of ADP used for each experiment was estimated on the base of adenine absorbance at 254 nm, corrected for the real content of ADP in each batch assayed enzymatically according to [16, 17]. The total content of $Mg^{2+}$ in mitochondria and mitoplasts was assayed by atomic absorption spectroscopy using acid extracts of the preparations.

## RESULTS

### $Mg^{2+}$ weakens restrictions imposed by the outer membrane on the access of metabolites into YVDAC1-depleted mitochondria while dissipation of $\Delta\psi$ exerts the opposite effect

To quantify the apparent restrictions observed in the accessibility of large ionic molecules to mutant B5 mitochondria [5, 6], concentrations half-saturating the bioenergetic effect of a given species (i.e. $K_{0.5}$) were determined. It was assumed that this parameter allows a conclusion about the permeation of molecules through the outer membrane, since they must reach the inner membrane to act effectively. In the case of ADP the calculation of $K_{0.5}$ includes complex events, comprising the efficiency of adenine nucleotide translocase and ATP synthase. The ability of yeast mitochondria to oxidise external NADH, independently of the presence of YVDAC1 (Table 1), provides a simpler approach. Since electrons from external NADH are accepted by the rotenone-insensitive and antimycin A-sensitive dehydrogenase located at the outer surface of the inner membrane [19], the access of NADH to the respiratory chain is restricted only by the outer membrane.

In mutant B5 mitochondria the value of $K_{0.5}$ for NADH oxidation, supporting the resting state in the absence of $Mg^{2+}$, was found to be about nine times higher than in wild type mitochondria (approx. 360 $\mu$M *versus* 40 $\mu$M; Fig. 1). Addition of 4 mM $Mg^{2+}$ stimulated NADH oxidation in both types of mitochondria (Table 1) but exerted different effects on $K_{0.5}^{NADH}$ values. While in the wild type there was

**Table 1. Rates of oxygen uptake and values of the respiratory control index in yeast mitochondria**

| | Wild type | | Mutant | |
|---|---|---|---|---|
| | $-Mg^{2+}$ | $+Mg^{2+}$ | $-Mg^{2+}$ | $+Mg^{2+}$ |
| Resting state | 38 ± 3 | 46 ± 4 | 27 ± 2 | 41 ± 3 |
| Phosphorylating state | 123 ± 8 | 138 ± 13 | 42 ± 3 | 120 ± 10 |
| Uncoupled state | 132 ± 10 | 150 ± 12 | 93 ± 8 | 130 ± 9 |
| Respiratory control | 3.2 ± 0.3 | 3.0 ± 0.2 | 1.5* ± 0.1 | 2.9* ± 0.2 |

Values of oxygen uptake are expressed in nmoles $O_2 \times min^{-1}$ per mg of mitochondrial protein. *The values of respiratory control index were calculated according to [18], referring to the resting state following the exhaustion of added ADP. However, when the resting state could not be achieved due to a large dose of ADP, respiratory control index values were calculated referring to the resting state before the pulse of ADP. Incubation conditions as in Materials and Methods. Additions: $Mg^{2+}$, 4 mM $MgCl_2$; NADH, 1.5 mM; ADP, 200 $\mu$M (wild type) or 800 $\mu$M (mutant).

practically no change of the $K_{0.5}^{NADH}$ value, in mutant mitochondria a significant decrease (over 30%) was observed. In the case of mutant mitochondria, the removal of the outer membrane caused a fundamental decrease in the value of $K_{0.5}^{NADH}$ and abolished the decreasing effect of $Mg^{2+}$, but was without any effect for the wild type ones. In consequence, the value of $K_{0.5}^{NADH}$ determined for mutant mitoplasts in resting state was almost the same as those calculated for wild type mitochondria and mitoplasts. In the uncoupled state a comparable increase of $K_{0.5}^{NADH}$ values for both types of mitochondria was observed. In the wild type the value of $K_{0.5}^{NADH}$ was higher by about 50% (60 $\mu$M) but still was not influenced by the presence of $Mg^{2+}$. In the case of mutant mitochondria the dissipation of $\Delta\psi$ caused an increase of $K_{0.5}^{NADH}$ values by about 65% in the absence of $Mg^{2+}$ and by about 55% in its presence (see also Fig. 2). Thus, for both types of mitochondria the values of $K_{0.5}^{NADH}$ depended on the rate of oxygen uptake and increased simultaneously with the transition from the resting to the uncoupled state. However, the effect was not observed for both types of mitoplasts. This points out that in the uncoupled state in both types of mitochondria the passage through the outer membrane is the limiting step in NADH accessibility.

The effective triggering of the fully active phosphorylating state in mutant mitochon-

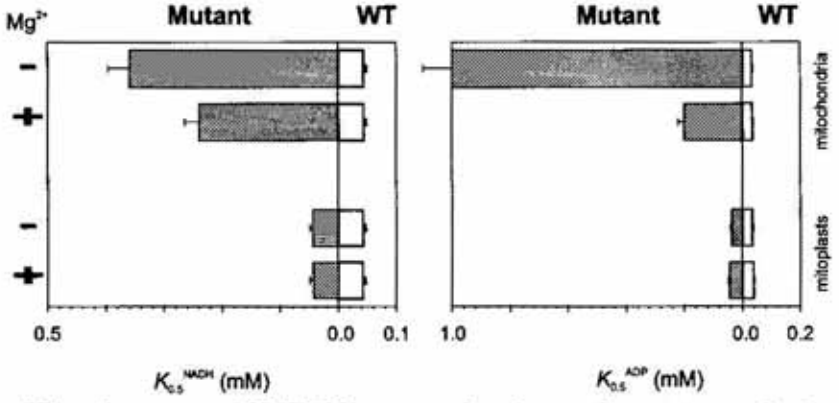


**Figure 1. Values of $K_{0.5}$ for external NADH in supporting the resting state respiration and for ADP in triggering the phosphorylating state, determined for wild type and YVDAC1-depleted mutant B5 mitochondria and mitoplasts.**

$K_{0.5}^{NADH}$ represents the concentration of external NADH supporting half-maximal oxygen uptake. $K_{0.5}^{ADP}$ represents the concentration of ADP yielding half-maximal stimulation of oxygen uptake taking place during transition from the resting to the phosphorylating state. Incubation conditions as in Materials and Methods; $Mg^{2+}$, 4 mM $MgCl_2$. Abbreviation: WT, wild type.

dria, which oxidised NADH in the presence of $Mg^{2+}$, required a much higher concentration of ADP than in wild type mitochondria; ($K_{0.5}^{ADP}$ approx. 200 $\mu$M and 40 $\mu$M, respectively, Fig. 1). In the absence of $Mg^{2+}$, the response to ADP in intact mutant mitochondria was severely hampered and the concentration of ADP yielding half-maximal stimulation of oxygen uptake ($K_{0.5}^{ADP}$) was found to be higher than 1000 $\mu$M. However, in mutant mitoplasts a dramatic decrease in the value of $K_{0.5}^{ADP}$ to the level observed for wild type mitochondria, accompanied by a loss of its $Mg^{2+}$ dependence, was observed. Therefore, the main component of the effect of $Mg^{2+}$ seems to occur at the level of the outer membrane. The important role of $Mg^{2+}$ in triggering the phosphorylating state in intact mutant B5 mitochondria could also be concluded from the values of $K_{0.5}^{Mg}$ calculated for the stimulation of NADH oxidation in all respiratory states. The value of $K_{0.5}^{Mg}$ was the same for both types of mitochondria in all states (about 1 ± 0.2 mM), except for the phosphorylating state in mutant mitochondria for which it was distinctly higher (2.8 ± 0.2 mM). The omission of EDTA from the isolation medium did not change the pattern of the stimulation of respiration due to $Mg^{2+}$. Moreover, the level of total magnesium was comparable in both wild type and mutant mitochondria (approx. 100 nmoles of $Mg^{2+}$ per mg of mitochondrial protein). These observations allow one to conclude that drastic efflux of $Mg^{2+}$ from mutant mitochondria did not take place during preparation. All these effects of $Mg^{2+}$ on NADH oxidation in both types of mitochondria in different respiratory states could also be observed when $Mg^{2+}$ was replaced by $Ca^{2+}$. The calculated values of $K_{0.5}^{Ca}$ for the stimulation of oxygen uptake in all respiratory states were about 40% lower than those for $Mg^{2+}$. Electrophoretic uptake of $Ca^{2+}$ was excluded as the observed effects persisted in the presence of ruthenium red, a known inhibitor of $Ca^{2+}$ uniport, which is consistent with the lack of an active $Ca^{2+}$ carrier in yeast mitochondria [20].

Thus, the outer membrane depleted of YVDAC1 constitutes a barrier in the passage of molecules into the intermembrane space. The barrier is partially abolished in the presence of $Mg^{2+}$ and seems to be more restrictive for ADP than for NADH.

**Restricted passage of molecules through the outer membrane in YVDAC1-depleted mutant mitochondria leads to competition between them for the passage**

Since some competition between molecules to pass through the outer membrane depleted of YVDAC1 has been suggested [6], this aspect of the passage was studied more carefully. Such competition could be defined as additional obstacles in the bioenergetic effects triggered by a given kind of molecule in the presence of another penetrating one. The existence of such obstacles imposed by carboxyatractylate and ADP on NADH transport to the intermembrane space of YVDAC1-depleted mitochondria can be concluded from the increase of $K_{0.5}^{NADH}$ values observed in their presence (Fig. 2). Due to an addition of 800 $\mu$M ADP, the value of $K_{0.5}^{NADH}$ determined in the not fully active phoshorylating state (in the absence of $Mg^{2+}$) was increased by about 45% when compared with that in the resting state. Thus, $K_{0.5}^{NADH}$ approached the value obtained in the uncoupled state, although the observed rate of oxygen uptake was much lower than in the latter (see Table 1). In the fully active phosphorylating state triggered by the same concentration of ADP in the presence of $Mg^{2+}$, the value of $K_{0.5}^{NADH}$ was only about 20% higher than that in the resting state, albeit the oxygen uptake was comparable to that in the uncoupled state. In the presence of 80 $\mu$M CATR, the value of $K_{0.5}^{NADH}$ in the resting state was increased by approx. 75% in the absence of $Mg^{2+}$ and by 60% in its presence. The highest increase in the value of $K_{0.5}^{NADH}$ (about 140%) resulted from the simultaneous application of ADP and CATR in the resting state and in the absence of $Mg^{2+}$ (not shown). In the uncou-

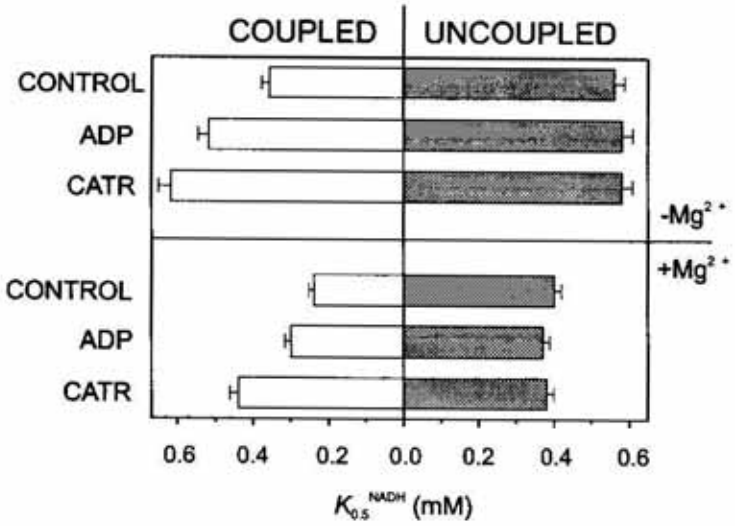


**Figure 2. Changes of $K_{0.5}^{NADH}$ values in YVDAC1-depleted mutant B5 mitochondria imposed by state of respiration and the presence of ADP and CATR.**

Incubation conditions as in Materials and Methods. Additions: $Mg^{2+}$, 4 mM $MgCl_2$; ADP, 800 $\mu$M; CATR, 80 $\mu$M.

pled state, regardless of the presence of $Mg^{2+}$, the effect of ADP and/or CATR on $K_{0.5}^{NADH}$ was abolished nearly completely (maximal increase about 10%) but in their presence the rate of oxygen uptake was distinctly lower (by about 30–35%). This apparent competition between NADH and ADP/CATR for the entry into the intermembrane space observed in mutant B5 mitochondria was not observed in wild type mitochondria.

Another kind of competition in mutant B5 mitochondria was that observed between ADP and CATR. Since CATR is considered as a non-competitive inhibitor of adenine nucleotide translocase, it was assumed that any significant lag in its action would be due to its delayed entry to the intermembrane space. Fig. 3A shows the levels of $\Delta\psi$ following the triggering of the phosphorylating state and subsequent addition of CATR recorded for both types of mitochondria. The traces obtained in the presence or absence of $Mg^{2+}$ for different concentrations of ADP and CATR were used to express the effectiveness of

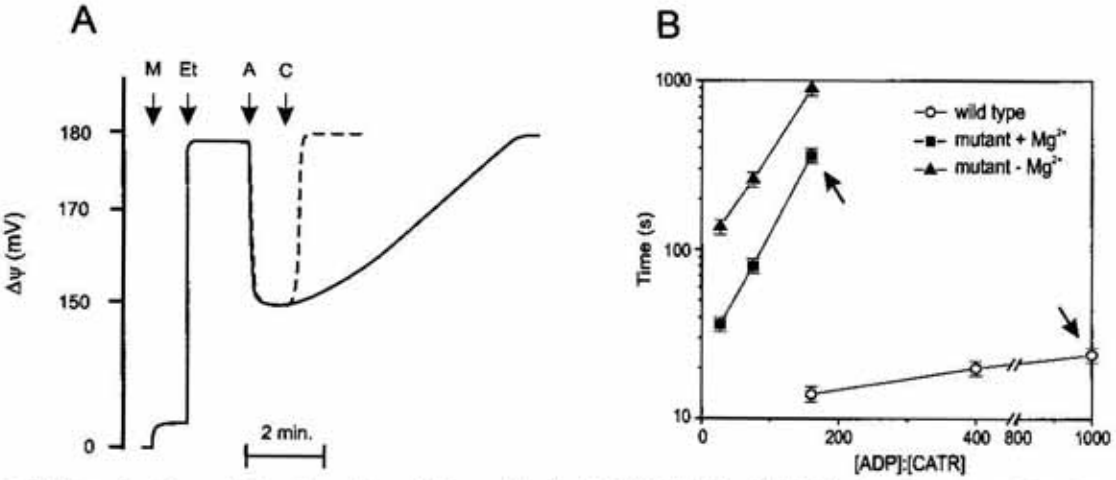


**Figure 3. Termination of the phosphorylating state by CATR in YVDAC1-depleted mutant B5 mitochondria as a function of the ratio of ADP and CATR concentrations.**

(A) Traces of the levels of $\Delta\psi$ recorded for both types of mitochondria in the presence of 4 mM $Mg^{2+}$. Incubation conditions as in Materials and Methods. Dashed line, wild type mitochondria; full line, mutant B5 mitochondria. Additions: M, 1 mg mitochondrial protein; Et, 20 mM ethanol; A, 1500 $\mu$M ADP; C, 1.5 $\mu$M (wild type) or 9.4 $\mu$M (mutant) CATR. (B) Calculated data. The ranges of ADP and CATR concentrations were 200–1500 $\mu$M and 1–80 $\mu$M, respectively. Ethanol (20 mM) was used as a respiratory substrate; $Mg^{2+}$, 4 mM $MgCl_2$. Values of five experiments are shown ± S.D. Arrows indicate ratios calculated from experiments shown in (A).

CATR action as a function of the ratio of ADP and CATR concentrations. For intact mutant mitochondria the CATR-supported recovery of $\Delta\psi$ from the not fully active phosphorylating state (in the absence of $Mg^{2+}$) to the resting state was delayed by an increasing the ADP/CATR ratio (Fig. 3B, triangles). The observed delay was only partially diminished in the presence of $Mg^{2+}$ (Fig. 3B, squares). In wild type mitochondria this apparent competition between ADP and CATR was only negligible, if any (Fig. 3B, circles).

## DISCUSSION

Here we described some properties of a transport system of the outer mitochondrial membrane supporting the viability of the yeast mutant B5, depleted of YVDAC1, on non-fermentable carbon source. A common feature of the system and YVDAC1 seems to be the dependence of the intrinsic capacity on the rate of mitochondrial respiration since in both types of mitochondria its acceleration in the uncoupled state results in an increase of $K_{0.5}^{NADH}$. Thus, one could conclude that both pathways restrict the traffic of NADH through the outer membrane under conditions leading to its faster transport through the membrane. Properties specific for the transport system functioning in the absence of YVDAC1 were inferred with the assumption that the differences observed between wild type and mutant B5 mitochondria ensue from the lack of YVDAC1 (as there are no crucial differences in this respect between the two types of mitoplasts).

Firstly, the system has lower capacity than YVDAC1, which can explain restricted access of metabolites to the inner membrane. The restrictions are reflected in increased values of $K_{0.5}^{NADH}$ for respiration and $K_{0.5}^{ADP}$ for triggering the phosphorylating state (Fig. 1) as well as in a longer time needed for an effective inhibition of the phosphorylating state by CATR at given concentration (not shown). Secondly, the system displays a preference towards cationic metabolites. In the presence of $Mg^{2+}$, respective values of $K_{0.5}$ are significantly lower and the action of CATR is faster. Moreover, for mutant B5 mitochondria (but not for the wild type ones) the value of $K_{0.5}^{Mg}$ in the phosphorylating state is distinctly higher than in other states (approx. 2.8 mM *versus* 1.0 mM). A specific requirement of $Mg^{2+}$ for proper activity of adenine nucleotide translocase and/or ATP synthase in intact mutant mitochondria can be excluded since in mutant mitoplasts the induction of the fully active phosphorylating state by ADP does not require the addition of the cation. Thus, the facilitating action of $Mg^{2+}$ (or $Ca^{2+}$) in the passage of the transported species through the outer membrane in the absence of YVDAC1 occurs beside an influence on the rate of electron flow (Table 1). The involvement of bivalent cations in the passage of molecules through the outer membrane could consist in screening of their negative charge. However, a direct modification of the properties of the system by the cations, rendering it less cation-selective, can not be excluded.

These properties postulated for the transport system of the outer membrane depleted of YVDAC1 are further supported by a competition observed between various compounds for the transport through the membrane. As can be concluded from Fig. 2, during coupled respiration in the absence of $Mg^{2+}$, ADP and CATR impose constraints on NADH accessibility, stronger in the case of CATR. On the other hand, the passage of CATR through the outer membrane, estimated by a progressive termination of the phosphorylating state, is delayed by increasing concentrations of ADP (Fig. 3B, triangles). NADH seems to have no effect on the accessibility of ADP and CATR in mutant B5 mitochondria as replacement of NADH by ethanol does not change the quantitative indexes of energetic transitions (not shown). Therefore, one could conclude that the capability of these molecules to compete with the others for the access to the inter-

membrane space of mutant B5 mitochondria results from their negative charge. However, in the presence of $Mg^{2+}$ the competitive influence exerted by CATR on the accessibility of NADH is only slightly weaker when compared to that observed in the absence of $Mg^{2+}$, while that imposed by ADP is strongly reduced in the presence of the cation. This difference between the effect of ADP and CATR probably results from nearly two times higher molecular mass of CATR, which suggests that the size of the transported species is also important for the process of competition. The presence of $Mg^{2+}$ also decreases the competition between ADP and CATR, although effective termination of the fully active phosphorylating state still depends on the concentration ratio of both species (Fig. 3B, squares). A slight increase of $K_{0.5}^{NADH}$ imposed by ADP and/or CATR during uncoupled respiration in mutant mitochondria (Fig. 2) is surprising in terms of competition and control step. Its understanding requires further studies. However, the distinctly lower rate of oxygen uptake observed for the mitochondria during the uncoupled state in the presence of ADP and CATR suggests some obstacle in the access of NADH to the intermembrane space.

Low capacity and preference towards cationic metabolites point to a channel being the main component of the transport system. This channel seems to have a diameter smaller than that of YVDAC1 and be controlled by $Mg^{2+}$ (as postulated in [10]) as well as by $Ca^{2+}$. So far only two channels distinct from YVDAC1 have been found in the outer membrane of yeast mitochondria, namely the channel identified in a YVDAC1-depleted mutant constructed by Dihanich *et al.* [4, 11] and PSC (peptide sensitive channel) described among others in the mutant B5, the object of present studies [21]. In reconstituted systems both of them display cationic selectivity and the conductance lower than that of YVDAC1. Moreover, the PSC is regarded to be identical with TOM40 [22, 23], the putative translocation pore of the outer membrane protein import machinery (TOM complex). Since the coincidence of PSC and the channel described by Dihanich *et al.* [4, 11] is strongly supported and their characteristics correspond to that of the putative channel postulated in this paper, one might conclude about the identity of all three. This hypothesis seems to be supported by the observation that in the absence of YVDAC1 the accumulation of fusion protein $b_2$-DHFR within the TOM complex restricts the access of external NADH into mitochondria [24, 25].

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