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THE RESPIRATION OF CELLS AND MITOCHONDRIA OF PORIN DEFICIENT YEAST MUTANTS IS COUPLED

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Summary: Several mutants of yeast lacking the porin gene have been found stable and viable on glucose or glycerol media. Ethanol-supported respiration of porin-free mutant and wild cells appeared equally coupled in vivo being similarly depressed by inhibitors of ADP/ATP translocase or of ATP synthase and stimulated by the uncoupler FCCP. The absence of porin in isolated mutant mitochondria hardly impaired the electron flux but increased the requirement for Mg^{2+} (or Ca^{2+}) and for ADP and carboxyatractylate concentrations necessary to drive effectively state 3 - state 4 and state 4 - state 3 transitions, respectively. The existence of another porin species, possibly controlled by bivalent cations, is postulated.

The mitochondrial porin is the major integral protein of the outer mitochondrial membrane, where it forms an oligomeric general diffusion pore or voltage-dependent anion channel (VADC), allowing the non-specific passage of low molecular-weight hydrophilic solutes in its open state [1]. An estimated diameter of approximately 2 nm and a defined exclusion limit of Mr 4,000 to 6,000 should make the channel a crucial device allowing the free passage of substrates and metabolites, vital for the energetic and metabolic functions of mitochondria, through the outer membrane. Nevertheless, the role of the porin in controlling the metabolism and physiology of mitochondria is not fully understood, although several regulatory properties of porin-formed channels, especially the dependence of the conductivity of channels formed by porins isolated from various organisms on membrane voltage have been described in the reconstituted systems [2]. In addition evidence was recently provided to prove that the closed state of the pore in mitochondria is impermeable for ATP and ADP [3]. One would expect the absence of porin from mitochondria to largely limit the uptake of charged molecules eg. ADP, ATP and the respiratory substrates, and to impose severe constraints on the efficiency of energy generation and especially of ATP formation in these organelles with a large impact on cell growth and physiology. Therefore several yeast mutants characterized by the phenotypic absence of the mitochondrial porin were constructed by disruption-deletion of the cloned porin gene [4-6]. Most of the mutants accumulate a large amount of a 86

Abbreviations: BA, bongkrekic acid; TBT, tributyltin Cl; CATR, carboxyatractylate; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone .

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kd protein and a possible relationship between this accumulation and the efficiency of mitochondrial respiration has been suggested [5]. In the present paper we report on the status of energy coupling in the cells and isolated mitochondria of this type of mutants. Some of the results have been briefly reported [7].

MATERIALS AND METHODS

Description of porin deficient mutants has been briefly reported previously [4, 7, 8] and is summarized in Table I. All of them, except R1 were unable to grow at 37°C on rich medium with glycerol as the carbon source (YPG) and accumulated a large amount of the 86 kd protein. B5 mutant was the only one that did not produce any detectable amount of truncated porin mRNA. Mutant R1 was isolated from the porin-free strain D9 on the basis of its ability to grow at 37°C on YPG plate. No accumulation of the 86 kd protein could be observed when total protein extract of R1 cells grown on YPG liquid medium was subjected to SDS gel electrophoresis [6]. Cultures were grown at 28°C on YPG and cells were collected at the late exponential phase of growth.

For the measurement of respiration the cells were washed three times in 30 mM KPi buffer pH 5 and starved for 10-20 hours with shaking at 28°C in the same buffer in the presence or absence of 30 μg oligomycin in 1 ml samples containing about 20 OD units of the cells. Cell respiration was measured oxygraphically at 25°C in 2 ml of 30 mM KPi buffer pH 5 to 7 or of 50 mM K/ citrate buffer pH 4.5 saturated with air (500 nAt O x ml^{-1}).

Mitochondria were isolated essentially the same way as in [9] from zymolyase-treated cells. The recovery of mitochondrial protein was not lower in mutant strains than in the wild ones.The degree of integrity of the outer mitochondrial membrane was measured with exogenous cytochrome c according to [10]. With this test both, wild type and mutant mitochondria were found intact in about 90%.

Mitochondria respiration was monitored at 25°C with a Rank oxygen electrode in the incubation medium (2.5 ml) saturated with air (440 nAt O x ml^{-1}) containing : 0.6 M mannitol, 5 mM KCl, 10 mM Hepes pH 6.9, 10 mM KPi, 4 mM $MgCl_2$, 0.2 % BSA (defatted), 0.8-1.6 mg mitochondrial protein.

RESULTS

In general, all of the porin-free mutants studied exhibited similar growth and respiratory characteristics. B5 mutant is given as the example because it has been already used in other studies [16].

Growth of B5 cultures

Although the cultures of the mutant B5 grew in 2 % glucose medium at the same rate as the parent strain DBY747 with doubling time about 2 hours at 28°C, they terminated the exponential phase of growth at approximately twice lower density than the wild strain (OD 10 and 18, respectively). Therefore, it was difficult to obtain the cells and mitochondria of B5 strain in a derepressed state. That

TABLE I. Characteristics of yeast porin mutants

Porin mutant	Isogenic wild type strain	Mutation	ts phenotype	86 kd protein
PH2	GRF18	HIS3 insertion at BclI site	+	+
D9	DBY747	EcoRV deletion	+	+
R1	DBY747	EcoRV deletion	-	-
B5	DBY747	EcoRV-NcoI deletion, URA3 insertion	+	+

was possibly the reason for low cytochrome level and cytochrome c oxidase activity observed in another porin-deficient mutant [5]. However, with the initial level of glucose in the medium lowered to 0.5 % both the wild and mutant cultures arrived at the stationary phase at approximately the same time and density (OD about 4) and derepression could be similarly obtained in both of them.

When inoculated from glucose into glycerol medium the mutant grew apparently slower than the wild strain, however, with no striking difference between the two strains in the short (3-5 hours) initial lag. In contrast to that of the parent strain the growth rate of B5 mutant was highly sensitive to pH of glycerol medium and was 4 times faster at pH 5.5 (adjusted with HCl) than at pH 6.7 of a non-adjusted medium. Therefore, at pH 5.5 of glycerol medium the doubling time of B5 culture (3.5 hours) was only slightly larger than that of the parent strain (3 hours). However, the pH value of glycerol growth medium did not influence either the temperature-sensitive (ts) growth phenotype on glycerol solid medium at 37°C or the final yield of B5 culture which reached the stationary phase at somewhat higher density (OD 3.5 to 4) than DBY747 culture did (OD 3). Moreover, at low pH no significant deficiency of growth was observed in this porin-deficient mutant when compared to the parent strain, neither any strikingly long adaptation time nor was any pregrowth on glycerol solid medium needed for B5 mutant to grow on liquid glycerol medium as efficient as the parent strain. This is in contrast to the growth characteristics of another porin-deficient yeast mutant [5] grown in a medium of not precised pH.

Respiration of cells

The ethanol supported oxygen uptake of starved glycerol-grown B5 cells was only 10-15 % lower than that of parent cells. The maximal rate of this respiration in the presence of an uncoupler FCCP changed somewhat with time, temperature and shaking rate during starvation and depended only slightly on pH of the incubation medium ; at pH 5.0 (25°C) it was of the order of 20-30 nAt O x min^{-1} x OD $unit^{-1}$ of cells. The optimal concentration of FCCP releasing respiratory control and yielding the highest respiratory rate depended on the strain : 1.2 μM for parent strain and 2.4 μM for B5 mutant, whereas the twofold higher FCCP concentrations inhibited cell respiration significantly.

The rate of cell respiration is controlled by the respiratory state of its mitochondria which, in turn, depends mainly on accessibility of substrates and ADP and fluctuates between the resting state 4 (high membrane potential $\Delta\Psi$, low O_2 uptake) and the phosphorylating state 3 (lower $\Delta\Psi$, high O_2 uptake). State 3 respiration is in turn limited by availability of ADP and the activity of the respiratory chain, ATPase and adenine nucleotide carrier. At a given level of respiratory substrate the maximal rate of O_2 uptake (i.e., the electron flow through the respiratory chain) could be revealed in the presence of an uncoupler (e.g. FCCP), collapsing $\Delta\Psi$ totally. This uncoupled respiration was usually very little higher than that of state 3 at saturating level of ADP. On the other hand the real state 4 with its low O_2 uptake could be experimentally imposed by inhibitors of adenine nucleotide carrier [e.g. bongkrekic acid (BA)] or ATP synthetase [e.g., oligomycin, tributyltin (TBT)]. Actual (native) cell respiration fluctuated between these two extremes being maintained at attenuated state 3 and mainly controlled by the level of cytosolic ADP or, more precisely, its accessibility to the inner mitochondrial membrane via porin of the outer mitochondrial membrane (control by Ca^{2+} cycling could be excluded as an active mitochondrial Ca^{2+} carrier is not present in yeast [11]). One could expect that in porin deficient mutants the ADP-dependency of respiration might be absent -if any $\Delta\Psi$ is generated at all - thus, the coupling of cell respiration could be severely hampered.

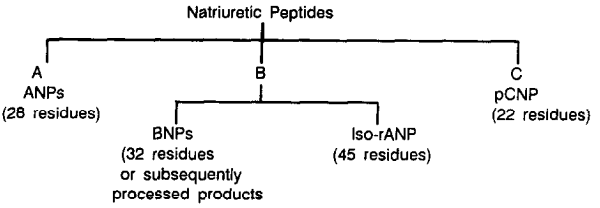

Figure 3. The relationship of rat Iso-ANP with other natriuretic peptides. The classification into three types of peptides is according to Sudoh et al. (7).

We have noticed potential regulatory DNA motifs within intronic sequences of iso-ANP (underlined in Fig. 2). Most conspicuous is a 20 nucleotide long alternating purine pyrimidine stretch in the first intron. Such an element has the potential of adopting a Z-DNA conformation and is often associated with transcriptional enhancers (22). Putative Z-DNA motifs have previously been reported at the 3' flanking regions of the rat and mouse ANP genes (17). A potential CCAAT element and its complementary form (ATTGG) (23) are also present 16 nucleotides downstream and 48 nucleotides upstream, respectively, from the putative Z-DNA element. In addition, DNA elements with high degree of homology (87.5%) with the consensus AP2 sequence (CCCCAGGC) (24) are also present in both introns. At the present moment we do not know the functional significance, if any, of these motifs with regard to expression of the rat iso-ANP gene. However, it is worth mentioning in this regard that a potential cis-acting regulatory element, namely, a glucocorticoid responsive element (GRE) has previously been reported within the second intron of the human ANP gene (19).

In reporting the cDNA sequence of rat BNP (iso-ANP) Kozima et al. (9) have observed nucleotide substitutions of C-to-A at three positions, without involving concomitant amino acid changes. Two of these sites are located in the 2nd exon, 14 nucleotides (involving the codon CGC for arginine) and 29 nucleotides (involving the ATC codon for isoleucine) upstream from the exon/intron junction (Fig. 2). The third site is located 24 nucleotides downstream from the stop codon, in the 3rd exon (nucleotide 1120, Fig. 2). We have noticed a T nucleotide in all three polymorphic sites of the rat iso-ANP, again without accompanying amino acid changes. Since two of the involved amino acid residues are located within the biologically important disulfide-bonded ring region, the selection pressure against their change is not surprising. What is surprising, however, is to find that the change at the third site, involving the 8th triplet (TCC) downstream from the stop codon, is also constrained as if to conserve a particular amino acid (serine) coded for by this triplet. Since there is an adjacent TGA triplet, 6 nucleotides downstream from this site, in phase with the reading frame, it is tempting to speculate that this triplet was perhaps functional previously as a stop codon of a longer peptide and that the presently functional stop codon resulted due to a C-to-A change of a previously functional serine (TCG) codon. In addition to these neutral polymorphisms, we have previously reported a Gln as well as a Leu residue at the penultimate position of iso-ANP (3,4). At the nucleotide level, we have observed the codon (TTG) for a leu residue, confirming the report of Kojima et al. (9). The discrepancy could be due to genetic variation among rats used for the atrial source of iso-rANP.

As discussed above, rat iso-ANP has a close structural relationship with BNPs from other mammalian species and yet has significant differences. We, therefore, view these two peptides as

either adenine nucleotide carrier with BA or mitochondrial ATPase with TBT (fig.1). The use of BA is more specific in view of no side-action either on the electron transport or the membrane potential but, due to penetration of only the undissociated form of BA through the membrane, is limited to a low pH (< 4.5) of the incubation medium. 12 μM BA was sufficient to shift cell respiration towards state 4 within 2 min while lower BA concentrations revealed a lag of several minutes. On the other hand the use of TBT was hampered by its complex mode of action. Sensitivity of S. cerevisiae respiration to trialkyltin was reported [12]. Tributyltin is one of the organic-tin compounds known as inhibitors of oxidative phosphorylation at the site of mitochondrial ATP synthetase [13].

For a given strain at pH 4.5, the values of state 4 respiration enforced by 10 μM BA or 20 μM TBT were almost the same while the maximal oxygen uptake released by FCCP was independent of the presence of these inhibitors. A very similar degree of inhibition of respiration could be obtained with respectively lower concentrations of TBT at pH 5 to 7, at which BA was not efficient (fig.1). Therefore, this degree of inhibition can be considered as a reliable reflection of a gap between the native attenuated phosphorylating state in the cells and the imposed state 4 and of the ability of ADP to control energetic transitions in situ. In this respect no difference seems to exist between the parent and B5 mutant cells (Table II). The same type of response of cell respiration to imposing state 4 and uncoupled state as above was observed in other mutants of DBY747 examined in the present study, i.e. D9 and R1, and in mutant PH2 and its parent strain GRF18 (fig.2). This suggests an efficient traffic of ADP and ATP between cytosolic and mitochondrial compartments through the mitochondrial membranes in the absence of porin.

Energy coupling in isolated mitochondria

It was found that in the presence of 8 mM ethanol neither the state 4 respiration nor the respiration fully uncoupled by valinomycin, measured in mitochondria from mutants lacking porin, were significantly

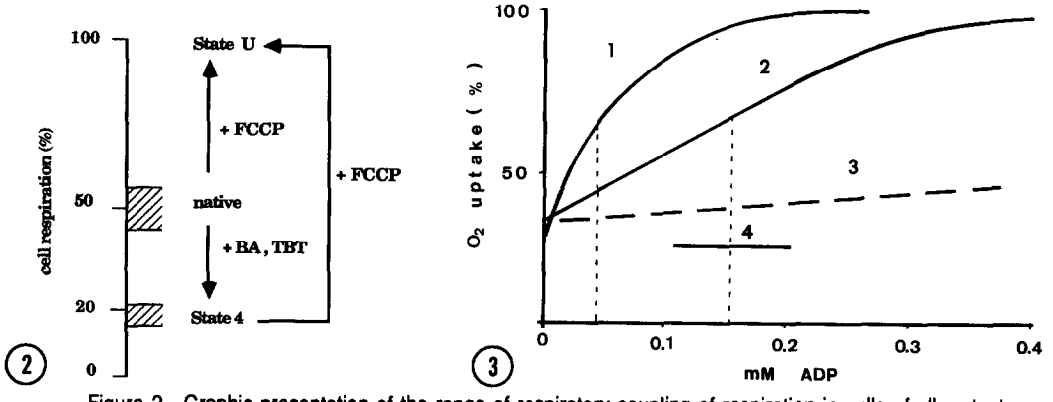

Figure 2 . Graphic presentation of the range of respiratory coupling of respiration in cells of all mutant examined. Conditions as in legend to fig.1. Crossed areas illustrate variations in three to six independent experiments for each mutant.

Figure 3 . Dependence of ethanol supported state 3 respiration of mutant and wild type mitochondria on ADP concentration. Wild type + Mg^{2+} (1); mutant + Mg^{2+} (2) and - Mg^{2+} (3) ; wild type and mutant + Mg^{2+} + CATR (4). Incubation conditions as in Methods; ethanol 8 mM; $MgCl_2$ 4 mM; CATR 1 and 15 μM in wild type and mutant mitochondria, respectively. 100 % O_2 uptake in the presence of valinomycin (0.1 mg x min^{-1} mitochondrial protein) was 220-300 nAt O x mg^{-1} protein for two wild strains and four mutants.

different from those in mitochondria from parent wild strains. Thus, the efficiency of the respiratory chain did not seem to be strongly influenced by the lack of porin. In contrast, however, there was a significant difference in the dependence of the initial state 3 oxygen uptake on the concentration of ADP added. While the curves of this dependence in wild strain mitochondria reveal a hyperbolic shape with an app.K_m about 50 μM ADP those in mitochondria from porin lacking mutants are more linear with an app.K_m about 160 μM ADP (fig. 3). K_m symbolizes here the ADP concentration supporting the half-maximal respiratory control of the respiration $(V_{state\ 3} - V_{state\ 4})_{0.5}$. Correspondingly, the course of return of state 3 to state 4 reflecting the decrease in external ADP concentration is sluggish on oxygen tracings in mutant mitochondria in contrast to a much sharper state 3-state 4 transition in mitochondria of the wild strains. This pattern of response caused serious difficulties in measuring the ADP:O ratio with small pulses of ADP in mutant mitochondria. However, with pulses of higher than 300 μM ADP the respiratory control index 3.0-3.5 and the ADP:O ratio 1.4-1.7 could be measured in mutant mitochondria, the values being close to those in mitochondria from the wild strains. (In S. cerevisiae the oxidation of ethanol, a substrate penetrating freely through the membranes, is coupled only at two phosphorylating sites).

While the omission of Mg^{2+} from the incubation medium did not change the response of oxygen uptake to the pulses of ADP and did not increase the apparent K_m^{ADP} for inducing state 3 respiration in wild strain mitochondria, the presence of external Mg^{2+} was necessary for such a response in mutant mitochondria. In fact, in spite of the presence of 200-400 μM ADP, only after the addition of 4 mM Mg^{2+} or Ca^{2+} could the reversible transition from the resting state to the phosphorylating one be observed and the ADP:O ratio measured in mutant mitochondria. While in wild strain mitochondria 1 μM CATR was efficient in the transition of state 3 into state 4 within half a minute as proved by the inhibition of oxygen uptake, 10-15 times higher concentration of this inhibitor was needed to drive such a transition in mitochondria of mutants lacking porin (data not shown).

DISCUSSION

The extensive and fast response of cell respiration to the inhibitors imposing state 4 and to the uncoupler (fig.2), almost identical in wild type and porin-free mutant cells, speaks strongly for the thight coupling and efficient traffic of adenine nucleotides between cytosolic and mitochondrial compartments in the absence of porin in situ, thus when the outer mitochondrial membrane is certainly intact. This statement is corroborated by the finding that in isolated mitochondria with largely intact outer membrane ADP could, in the presence of bivalent cation, effectively trigger the phosphorylating state, and thus could be transported though at much higher K_m^{ADP} than in the wild type mitochondria.

A much higher increase in external CATR concentration required in mutant mitochondria to drive an effective state 3-state 4 transition (by inhibition of adenine nucleotide translocase) than that in the ADP concentration needed for the maximal state 3 respiration might be due to CATR having a 1.8 times higher molecular weight (MW 760) and one more negative charge than ADP (MW 480). As CATR inhibition of the translocase is not competitive with respect to ADP, one can assume some competition between both molecules to pass through the outer membrane. In isolated mitochondria this passage was apparently dependent on the presence of bivalent cations and this might be interpreted either in terms of

screening of the negative charges of these molecules to allow them to pass through another pore of less anionic selectivity than that of the major porin or in terms of controlling the open state of this pore by cations. Apparently, the concentration of Mg^{2+} or other multivalent cations in the cytoplasm of mutant cells is favorable enough in vivo to enable the access of ADP to mitochondria as suggested by an efficient growth of the cells on a nonfermentable carbon source and by the same pattern of respiratory coupling in mutant and wild cells.

When grown on nonfermentable carbon sources the mutants (D9, PH2 and B5) except R1 exhibit a clear ts phenotype at 37°C and accumulate within the cells a large amount of a 86 kd protein [6]. The same was previously reported in a similar porin-free mutant [5] which, however, revealed a dramatic lesion of the respiratory chain activity and needed a three days adaptation period before growth on glycerol could be reassumed by about 25 % of the initially plated cells. It should be emphasized that the mitochondria of a porin-free suppressor strain R1, which does not accumulate in vivo the 86 kd protein, revealed a similar electron transport chain activity and the same apparent limitation of the accessibility of ADP and CATR as the mitochondria of those porin-free mutants (D9, PH2 and B5), which accumulate this protein. Moreover, no striking impairement of electron transport chain activity was observed either in cells or in isolated mitochondria of any of the porin-free mutants compared to those of the parents strains. In view of these findings the suggestion of a relationship between the accumulation of 86 kd protein and the efficiency of mitochondrial respiration based on the data obtained with another porin-free mutant [5] can not be generalized. The 86 kd protein in yeast has been recently identified as a major coat protein of virus-like particles [14]. The lesion of respiratory activity resulting from the absence of this protein in mutant deficient in the genome of these particles does not seem to be relevant to the present work.

In conclusion, the results suggest similar fluxes of ADP in porin-free mutant and in parent strain cells and prove the penetration of ADP and CATR, in the presence of Mg^{2+}, through the outer membrane of isolated mitochondria deficient in the major porin. As the passive transport of these large charged molecules through the lipid bilayer of the outer membrane seems rather unlikely the existence of another porin species, possibly controlled by bivalent cations, is postulated.

While this conclusion bases on the performance of outer mitochondrial membrane permeability in native or near-native state (in vivo and in vitro) it gains support from the recent reconstitution studies. The detergent-solubilized outer mitochondrial membrane proteins of another yeast porin-free mutant incorporated into lipid bilayer membranes were found to form transient pores which have the characteristics of general diffusion pores and reveal some cationic selectivity [15]. The existence of electrically similar, slightly cationic channels in mitochondria of porin-free B5 mutant and its isogenic wild type strain can be also deduced from the results of the measurements performed with microelectrodes [17] sealed at the tip with proteoliposomes derived from isolated yeast mitochondria [16].

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