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MINI-REVIEW

Pores from Mitochondrial Outer Membranes of Yeast and a Porin-Deficient Yeast Mutant: A Comparison

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Abstract

Reconstitution experiments were performed on lipid bilayer membranes in the presence of purified mitochondrial porin from yeast and of detergent-solubilized mitochondrial outer membranes of a porin-free yeast mutant. The addition of the porin resulted in a strong increase of the membrane conductance, which was caused by the formation of ion-permeable channels in the membranes. Yeast porin has a single-channel conductance of 4.2 nS in 1 M KCl. In the open state it behaves as a general diffusion pore with an effective diameter of 1.7 nm and possesses properties similar to other mitochondrial porins. Surprisingly, the membrane conductance also increased in the presence of detergent extracts of the mitochondrial outer membrane of the mutant. Single-channel recordings of lipid bilayer membranes in the presence of small concentration of the mutant membranes suggested that this membrane also contained a pore. The reconstituted pores had a single-channel conductance of 2.0 nS in 1 M KCl and the characteristics of general diffusion pores with an estimated effective diameter of 1.2 nm. This means that the pores present in the mitochondrial outer membranes of the yeast mutant have a much smaller effective diameter than "normal" mitochondrial porins. Zero-current membrane potential measurements suggested that the second mitochondrial porin is slightly cation-selective, while yeast porin is slightly anion-selective in the open state but highly cation-selective in the closed state. The possible role of these pores in the metabolism of mitochondria is discussed.

Key Words: Yeast; yeast mutant; mitochondrial porin; mitochondrial outer membrane; lipid bilayer; ion-channel.

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Introduction

The mitochondrial outer membrane of a variety of eukaryotic cells contains a pore-forming protein termed mitochondrial porin. This is responsible for the exchange of water-soluble metabolites across the mitochondrial outer membrane (Colombini, 1979; Zalman *et al.*, 1980; Roos *et al.*, 1982; Freitag *et al.*, 1982; Benz, 1985). Mitochondrial porins from different eukaryotic cells have been studied in detail (Schein *et al.*, 1976; Colombini, 1980; Lindén *et al.*, 1982; De Pinto *et al.*, 1986). The outer membrane of yeast mitochondria contains a major protein with a molecular mass of 30 kDa, which constitutes at least one-fifth of the protein mass of the membrane (Riezmann *et al.*, 1983; Gasser and Schatz, 1983; Mihara *et al.*, 1982). This protein was isolated and purified by standard methods (Ludwig *et al.*, 1982). Reconstitution experiments on lipid bilayers defined its function as an ion-permeable channel with a single-channel conductance of 0.42 nS in 0.1 M KCl (Ludwig *et al.*, 1988). Yeast porin is encoded by a nuclear gene and synthesized on cytoplasmic ribosomes without leader sequence (Mihara *et al.*, 1982; Gasser and Schatz, 1983). Its structural gene has been cloned and sequenced (Mihara and Sato, 1985). Its amino acid composition is not particularly hydrophobic, which indicates that arrangement of the polypeptide chain in secondary, tertiary, and quaternary structure is responsible for the activity of the protein as an intrinsic membrane protein. Disruption of the porin gene resulted in a viable yeast mutant whose growth rate on glycerol recovers to approximately half that of wild type cells after some time of adaptation (Dihanich *et al.*, 1987).

In this paper we describe reconstitution experiments with yeast porin, isolated and purified from the mother strain of a porin-free mutant according to a well-established method (Ludwig *et al.*, 1988). It is similar to the porin reported earlier and isolated from another strain of yeast (Ludwig *et al.*, 1988). It has the characteristics of a general diffusion pore with an effective diameter of 1.7 nm in the open state. Yeast porin from strain D273-10B is slightly anion-selective in the open state but becomes cation-selective after voltage-dependent closure. Furthermore, we studied the effect of detergent-solubilized mitochondrial outer membranes of the porin-free yeast mutant D273-10A on lipid bilayer membranes in more detail and compared the pore properties with those of the porin. As shown previously (Dihanich *et al.*, 1989), membrane-active material spontaneously inserted from detergent-solubilized outer membranes of the mutant into lipid bilayer membranes. Single-channel analysis indicated that the detergent-solubilized material formed transient pores with a single-channel conductance of 2.0 nS in 1 M KCl. The reconstituted pore had the characteristics of a general diffusion pore with an estimated effective diameter from the single-channel data of about 1.2 nm. This result indicates that the mitochondrial outer membrane

of the porin-free yeast mutant is not simply leaky but contains a porin-like channel.

Materials and Methods

Culture of the Yeast Cells and Isolation of Yeast Mitochondria

The cells of *Saccharomyces cerevisiae* strain HR 125-5B (MAT *a* gal2 his3 his4 leu2 trp1 ura3) and its porin-free mutant HR 125-5A (Dihanich *et al.*, 1987, 1989) were grown on full medium containing 1% yeast extract, 2% peptone, and 2% glucose or 3% glycerol, converted to spheroplasts, homogenized, and the mitochondria isolated therefrom as described previously (Daum *et al.*, 1982). The porin-free mutant was preadapted to glycerol on solid-rich medium before incubation of liquid cultures, since adaptation in liquid culture is nearly impossible.

Isolation of Mitochondrial Outer Membrane

The isolation of mitochondrial outer membranes (OM) was performed as has been described previously (Riezmann *et al.*, 1983), with the exception that the discontinuous sucrose gradient centrifugation was omitted. The post-mitochondrial supernatant was directly layered over a linear sucrose gradient ranging from 1.6 to 0.85 M sucrose. After centrifugation for at least 13 h at 100,000 × *g*, the OM band was recognizable as the uppermost band and collected by puncturing the tube from the side. The presence of OM proteins and possible contamination with mitochondrial inner membrane (IM) was tested by immune blotting and incubation with antibodies against the 70-kDa and the 33-kDa protein of the OM as well as cytochrome *c* oxidase subunit IV of the inner membrane.

Isolation and Purification of Yeast Porin

The mitochondrial porin from yeast strain strain HR 125-5B (MAT *a* gal2 his3 his4 leu2 trp1 ura3) was isolated from whole mitochondria essentially as has been published previously (De Pinto *et al.*, 1987).

Membrane Experiments

The method used for black lipid bilayer experiments has been described previously in detail (Benz *et al.*, 1978). The apparatus consisted of a Teflon chamber with two aqueous compartments. The circular holes in the wall between the two compartments had an area of either 2 mm^2 (for the macroscopic conductance measurements) or about 0.1 mm^2 (for the single-channel

experiments). Membranes were formed by painting onto the holes a 1% (mass/vol.) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, Alabama) in *n*-decane. The temperature was kept at 25°C throughout. All salts and buffers were analytical grade and obtained from Merck (Darmstadt, F.R.G.). The aqueous solutions were unbuffered and had a pH around 6 if not indicated otherwise. Voltage was applied using a pair of calomel electrodes with salt bridges. In the case of single-channel measurements, the membrane current was amplified by a Model 427 amplifier (Keithley, Cleveland, Ohio), monitored on a storage oscilloscope (Tektronix, Beaverton, Oregon, model 5115), and recorded on a strip chart or a tape recorder. The macroscopic conductance measurements were performed with a Keithley 610 C electrometer. Zero-current membrane potentials were measured with the same instrument 5–10 min after the application of a salt gradient across the membranes (Benz *et al.*, 1979).

Results

Incorporation of the Yeast Porin into Lipid Bilayer Membranes and Single-Channel Analysis

When the 30-kDa protein isolated from the yeast strain was added in small quantities (10–100 ng/ml) to the aqueous solutions bathing a lipid bilayer membrane, the specific conductance of the membrane increased by several orders of magnitude with an initial rapid increase from 15–20 min followed by an increase at much smaller rate (Benz, 1985). The membrane activity of yeast porin was observed regardless of whether the protein was added to only one side or to both sides of the membrane. The addition of the detergent Genapol X-80 alone in a similar concentration to that used in the presence of the porin did not lead to any appreciable increase in the membrane conductance.

The addition of smaller amounts of the porin from yeast to lipid bilayer membranes having small surface areas (0.1 mm^2) allowed the resolution of step increases in conductance (Fig. 1A). Most of the conductance steps were directed upward, and closing steps were only rarely observed at transmembrane potentials of 5–10 mV. Figure 2A shows a histogram of 136 conductance steps in 1 M KCl at a membrane potential of 10 mV. The most frequent value for the single-channel conductance of the yeast porin was 4 nS. The channel in the mitochondrial outer membrane of yeast was permeable for a variety of different ions. Table I shows the single-channel conductances in the presence of different salt solutions. Although there existed a considerable influence of the salt on the pore conductance, the ratio Λ/σ varied by less

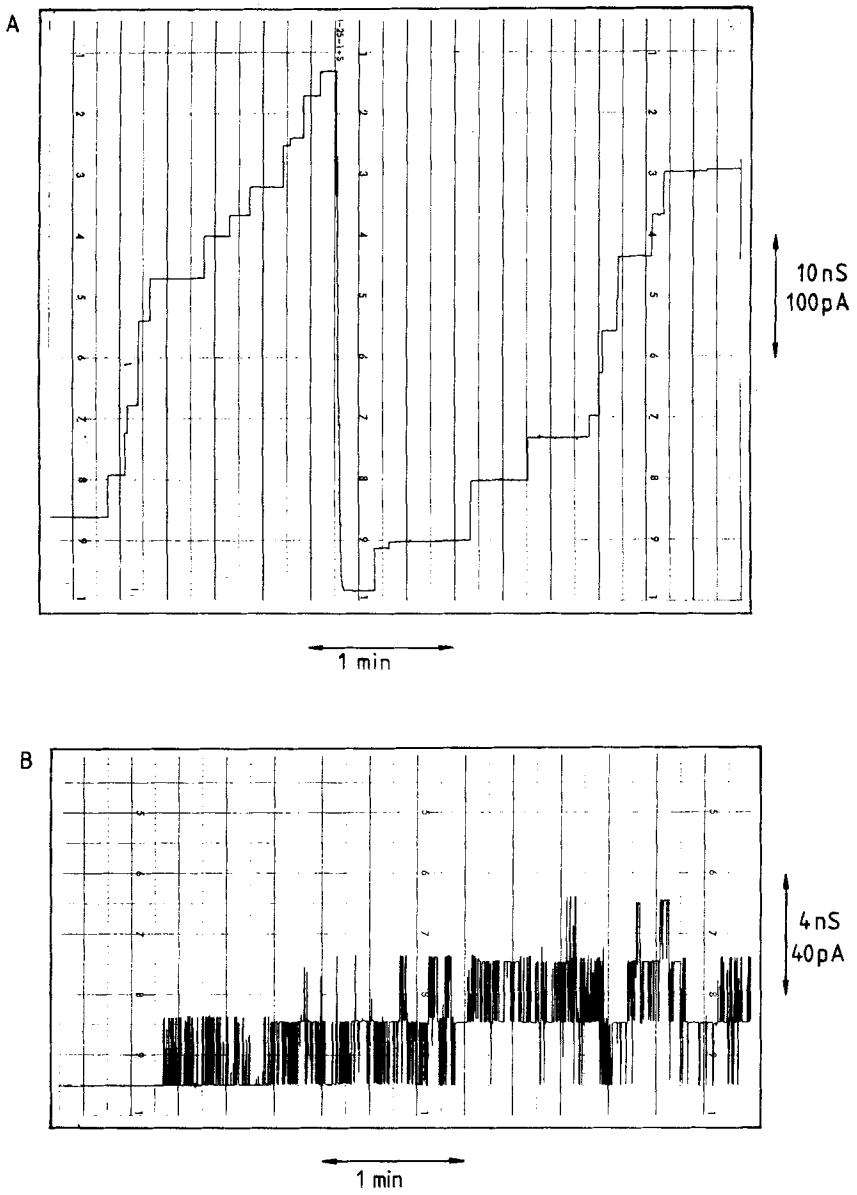

Fig. 1. Single-channel recording of diphytanoyl phosphatidylcholine/*n*-decane membranes after the addition of 5 ng/ml yeast porin isolated from strain HR 125-5B (A) and of 100 ng/ml mitochondrial outer membranes of the porin-free yeast mutant HR 125-5A (B) to the aqueous phase. The aqueous phase contained 1 M KCl, pH 6. The applied voltage was 10 mV; $T = 25°C$.

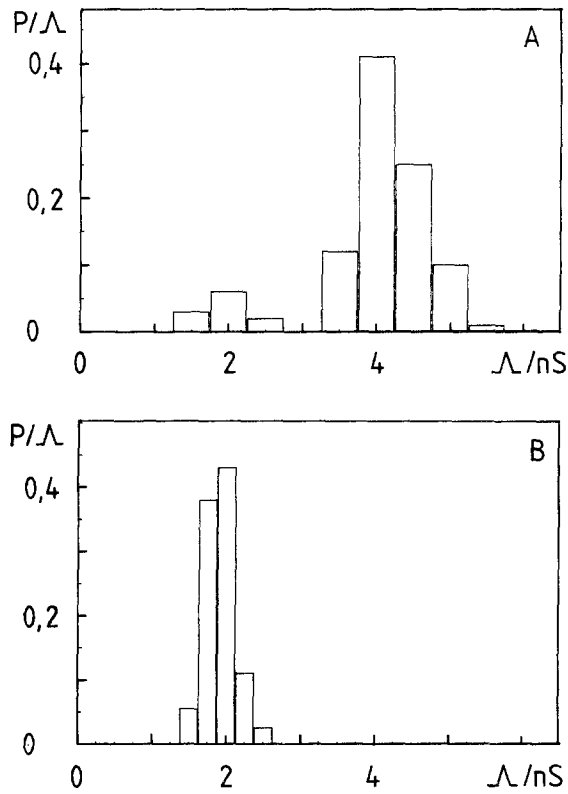

Fig. 2. Histogram of conductance fluctuations observed with membranes of diphytanoyl phosphatidylcholine/*n*-decane in the presence of yeast porin isolated from the yeast strain HR 125-5B (A) and of mitochondrial outer membranes from the porin-deficient yeast mutant HR 125-5A (B). The aqueous phase contained 1 M KCl. The applied voltage was 10 mV. The mean value of all conductance steps was 4.2 nS for 136 single events in the case of the yeast porin (A) and 2.0 nS in the case of the mutant membranes (B; 350 single events); V_m = 10 mV; T = 25°C.

than a factor of 2 (data not shown), i.e., the ions seemed to move inside the pore in a manner similar to the way they move in an aqueous environment.

Properties of the Closed State of Yeast Porin

At higher transmembrane potentials the closing events became more and more frequent. Furthermore, the closing events had in almost all cases a smaller amplitude than the initial on-steps. This result indicated that the yeast porin switched to substates at high voltages with an exponential time course (Ludwig *et al.*, 1988). The single-channel conductance of the closed state was investigated in detail to get some insight into its ion selectivity. The experiments were performed in the following way: membranes were formed

Table I. Average Single-Channel Conductance of Yeast Porin and the Pore in the Mitochondrial Outer Membrane of the Yeast Mutant in Different Salt Solutions of Concentration c^a

Salt	c (M)	Λ (nS)	
		Yeast porin	Pore from outer membrane of mutant mitochondria
KCl	0.01	0.050	0.022
	0.1	0.42	0.21
	0.3	1.3	0.55
	1	4.2	2.0
	3	10	4.9
NaCl	1	3.3	1.5
LiCl	1	3.2	1.2
KCH_3COO	1	2.6	1.4
$MgCl_2$	0.5	2.0	1.1

[a]The aqueous salt solutions contained either 5 ng/ml porin or 50–100 ng/ml mitochondrial outer membrane of the yeast mutant dissolved in 0.1% Genapol X-80; the pH was between 6.0 and 7.0. The membranes were made from diphytanoyl phosphatidylcholine/*n*-decane; $T = 25°C$; $V_m = 10$ mV. Λ was determined by recording at least 100 conductance steps and averaging over the distribution of the values.

Table II. Average Single-Channel Conductance of the Open and Closed State of Yeast Porin in Different 0.5 M Salt Solutions[a]

Salt	Open state (nS)	Closed state (nS)
KCl	2.3	1.3
K-MES	0.95	0.80
Tris-Cl	1.5	0.20

[a]The pH of the aqueous salt solutions was adjusted to 7.2. The membrane voltage was 40 mV; $T = 25°C$. The single-channel conductance of the closed state was calculated by subtracting the conductance of the closing events from the conductance of the initial opening of the pores. The data correspond to the mean of at least 50 single events.

in salts composed of cations and anions of different aqueous mobility. A transmembrane potential of 30 mV was applied to the membrane, and porin was added in a small concentration (5 ng/ml). The open state of the pore had under these conditions only a limited lifetime, which means that it was possible to measure the single-channel conductance of the closing events from those of the open state. Table II shows the results of this type of measurement obtained for three different salts composed of anions and cations of different mobility in the aqueous phase. The single-channel conductance of the closed state of the pore for Tris-HCl was considerably smaller than for K-MES, despite a similar aqueous mobility of K^+ and Cl^-. This result suggested that the closed state of the yeast porin is cation-selective.

Reconstitution of the Mitochondrial Outer Membrane of the Yeast Mutant

So far it was not clear if the mitochondrial outer membrane of the yeast mutant was simply leaky or if it contained another pore (Dihanich *et al.*, 1987). To test both possibilities, we dissolved mitochondrial outer membrane of the yeast mutant in 0.1% Genapol X-80 or Triton X-100 at a final concentration of 1 mg/ml. This solution was diluted 200-fold by adding it to the aqueous phase bathing black lipid bilayer membranes. Surprisingly, the addition of the outer membranes of the mutant had a strong effect on the conductance of the lipid bilayer membrane, and it increased by several orders of magnitude with a similar time course as described above for the porin (Dihanich *et al.*, 1989). The same effect was observed irrespective of whether the outer membranes were added to one or both sides of the artificial lipid bilayer membranes. The addition of detergent alone at the same concentrations (i.e., in control experiments) had only a minor influence on the membrane conductance. This result indicated that the conductance increase is caused by the presence of the outer membranes and not by the addition of the detergent (i.e., by an unspecific artifact).

Single-Channel Experiments with the Mutant Pore

The result described above could either mean that membrane-active material without any channel-forming capability was incorporated into the artificial bilayers or that the outer membrane of the yeast mutant contains a pore. To answer this question, we performed single-channel experiments. Small amounts of outer membrane of the mutant (50–100 ng/ml) were added to membranes of small surface (0.1 mm^2). Figure 1B shows a single-channel recording in the presence of the mitochondrial outer membrane of the yeast mutant at a voltage of 10 mV. The observed single-channel fluctuations had a limited lifetime (mean lifetime about 2 s) and usually decayed back to the baseline. This means that the presence of outer membrane of the mutant resulted in the formation of pores which showed an opening–closing reaction. Note that yeast porin is preferentially open at 10 mV (compare Figs. 1A and 1B). Figure 2B shows histograms of the conductance fluctuations observed under the conditions of Fig. 1B. Comparison of both histograms given in Figs. 2A and 2B indicated that the single-channel conductance of the pore from the mutant is about half of that of the normal porin.

The single-channel conductance was found to be a linear function of the bulk aqueous conductance (Table I). Single-channel experiments were also performed with salts containing ions other than K^+ and Cl^-. These experiments were done to study the ionic selectivity of the mutant mitochondrial pore. First chloride was replaced by acetate (Table I). This change had only little influence on the conductance of the mutant mitochondrial pore. The

influence of the cations on the single-channel conductance was more substantial, which is consistent with the assumption that the mutant mitochondrial pore is cation selective. Furthermore, the ionic selectivity followed the mobility sequence of the ions in the aqueous phase, which means that the mutant mitochondrial pore is a wide water-filled channel without a small selectivity filter (i.e., a binding site) (Benz, 1985). The pore from the mitochondrial outer membrane of the mutant was voltage-independent up to voltages of about 100 mV. This result represents another clear difference between this pore and yeast porin.

Ionic Selectivity of Yeast Porin and of the Pore of the Mitochondrial Outer Membrane of the Yeast Mutant

The ionic selectivity of yeast porin and the pore reconstituted from the outer membrane of the yeast mutant was measured by using zero-current membrane potential measurements in the presence of a KCl gradient. A tenfold KCl gradient across a membrane in which about 500 pores were incorporated resulted in an asymmetry potential of about -8 mV at the more dilute side for the yeast porin and of about 10 mV (positive at the more diluted side) for the pore reconstituted from the mitochondrial outer membrane of the yeast mutant. This result indicated preferential movement of anions over cations for yeast porin, and the opposite for the mutant pore at neutral pH. The analysis of the zero-current membrane potential using the Goldman–Hodgkin–Katz equation (Benz *et al.*, 1979) showed that the ratio of the anion permeability P_a divided by the cation permeability P_c was about 1.4 for the porin and about 0.60 for the pore from the mutant. This result suggested a small cation selectivity of the pore from the outer membrane of the porin-free yeast mutant in the case of the equally mobile potassium and chloride ions while the porin was slightly anion selective, which is in agreement with results obtained earlier (Ludwig *et al.*, 1988).

Discussion

In this study we have shown that we are able to reconstitute pore-forming proteins from the mitochondrial outer membranes of the yeast *Saccharomyces cerevisiae* and a yeast mutant which has lost the normal porin by gene disruption (Dihanich *et al.*, 1987). Control experiments demonstrated that the formation of both pores is not simply an artifact caused by the interaction of different detergents with the artificial membranes. The major protein component of the mitochondrial outer membrane of yeast was isolated and purified to homogeneity (Ludwig *et al.*, 1988). This protein

appears to be firmly integrated into the outer membrane. The isolated protein has an apparent molecular weight of 30 kDa on an SDS-electrophoretogram which is very close to the molecular weight as derived from the sequence (Mihara and Sato, 1985). The molecular weight of the protein responsible for the pores reconstituted from the mutant membranes is not known at present (Dihanich *et al.*, 1989). The studies on the incorporation of the purified protein into artificial lipid bilayer membranes define the function of both proteins as pore- or channel-forming components. Reconstitution at very low protein concentrations in the aqueous phase shows an increase of the membrane conductance in distinct steps. The single-channel conductance is about 4.2 nS in 1 M KCl for yeast porin, whereas the single-channel conductance of the mutant pore is about half of that value, which suggests that its cross section could be smaller.

The channels in the mitochondrial outer membranes of yeast and the yeast mutant were permeable for a variety of different ions. Table I shows the single channel conductances of both pores in the presence of different salt solutions. Although there existed a considerable influence of the salt on the pore conductance, the ratio Λ/σ varied by less than a factor of 2, i.e., the ions seemed to move inside both pores similarly to the way they move in an aqueous environment. This finding allowed a rough estimate of the effective diameter of the pores. Assuming that they are filled with a solution of the same specific conductivity σ as the external solution and assuming a cylindric pore with a length l of 6 nm [which is very likely according to electron microscopic studies of the porin pores of *Neurospora crassa* (Mannella and Frank, 1984)], the average pore diameter $d\,(=2r)$ can be obtained according to the equation

$$\Lambda = \sigma\pi r^2/l \tag{1}$$

The effective diameters of yeast porin and the mutant pore were estimated from their average single-channel conductances in 1 M KCl (Λ = 4.2 and 2.0 nS, respectively) to be about 1.7 and 1.2 nm (σ = 110 mS/cm).

A diameter of the mutant pore of 1.2 nm would allow the passage of molecules up to a molecular weight of about 800 Da (Benz, 1985). ATP and ADP have smaller molecular weights than 800 Da and could pass the porin in the outer membrane of the mutant mitochondria. On the other hand, the pore was found to be cationically selective, which represents a diffusion hindrance for the passage of anionic metabolites of mitochondria. It is interesting to note that isolated mitochondria of another porin-free yeast mutant (Guo and Lauquin, 1986) show a reduced respiration activity under normal conditions. This low respiration rate can be increased if 4 mM Mg^{2+} or 4 mM Ca^{2+} were added to the medium (Michejda and Lauquin, personal communication). This finding is consistent with our data because the porin

of the mutant is cationically selective, while the normal yeast porin has a small preference for anions in the "open" state (Ludwig *et al.*, 1988; Dihanich *et al.*, 1989). Large anionic solutes have definitely a smaller permeability through the outer membrane of the mutant. The addition of Mg^{2+} or Ca^{2+} to the media may lead to the formation of complexes between the anionic solutes and Mg^{2+}, thus masking the negative charges and increasing the permeation of the anionic solutes through the outer membrane.

The porin-free mutant needs some time to adapt to growth on glycerol-containing media (Dihanich *et al.*, 1987). This could mean that the new porin was induced during adaptation. This is, however, not very likely because of the lack of a similar adaptation process in other porin-free yeast mutants (Guo and Lauquin, 1986; Michejda and Lauquin, personal communication). Furthermore, we did not observe an additional band in the protein pattern of the outer membrane of the mutant and its parent strain (Dihanich *et al.*, 1989). This observation suggests that the pore may also be present in wild-type strains. An 86-kDa protein induced during adaptation to glycerol of the porin-deficient mutant does not have any pore-forming activity (data not shown) and, moreover, has recently been identified as the major coat protein of viruslike particles (Dihanich *et al.*, unpublished results). So far, it was impossible for us to clearly relate the new porin to one of the bands of the protein pattern of the outer membrane of wild type yeast and its mutant (Dihanich *et al.*, 1989).

In previous publications (Roos *et al.*, 1982; Benz, 1985; Brdiczka *et al.*, 1986) we have suggested that the closed state of the mitochondrial porin may be part of the control of mitochondrial metabolism. This suggestion would mean that the outer membrane restricts mitochondrial metabolism as has been shown for an inhibitor of the outer membrane pore (König *et al.*, 1977; Benz *et al.*, 1988). In fact, yeast porin is cationically selective in the closed state, and the yeast mutant has a reduced growth rate combined with a limited permeability of the mitochondrial outer membrane (Michejda and Lauquin, personal communication). These facts provided clear evidence that the mitochondrial metabolism could be controlled by the outer membrane pore. Furthermore, they agree with recent results that a synthetic polyanion could force rat liver porin into the closed state. This state is impermeable for adenosine triphosphate (ATP) and adenosine diphosphate (ADP) because the adenylate kinase activity could be blocked in a reversible way (Benz *et al.*, 1988). The discovery of a new pore in the mitochondrial outer membrane of yeast and the possibility of the presence of similar pores and also of others (Tedeschi *et al.*, 1987) in mitochondrial outer membranes of eukaryotic cells indicates that the function of this membrane is still not fully understood at present and deserves further investigations.

Acknowledgments

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