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Identification of two general diffusion channels in the outer membrane of pea mitochondria

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Reconstitution experiments were performed on lipid bilayer membranes in the presence of detergent solubilized mitochondrial membranes of pea seedlings (*Pisum sativum*). The addition of the detergent-solubilized material to the membranes resulted in a strong increase of the membrane conductance. To identify the proteins responsible for membrane activity the detergent extracts were applied to a hydroxyapatite (HTP) column and the fractions were tested for channel formation. The eluate of the column contained a protein which migrated as a single band with an apparent molecular mass of 30 kDa on SDS-PAGE. This channel was identified as the porin of pea mitochondria since it formed voltage-dependent channels with single-channel conductances of 1.5 and 3.7 nS in 1 M KCl and an estimated effective diameter of about 1.7 nm. Further elution of the column with KCl containing solutions yielded fractions which resulted in the formation of transient channels in lipid bilayer membranes. These channels had a single-channel conductance of 2.2 nS in 1 M KCl and had also the characteristics of general diffusion pores with an estimated effective diameter of 1.2 nm. Zero-current membrane potential measurements suggested that pea porin was anion-selective in the open state. The selectivity of the second channel was investigated by the measurement of the reversal potential. It was also slightly anion-selective. Its possible role in the metabolism of mitochondria is discussed.

Introduction

The mitochondrial outer membrane of a variety of eukaryotic cells contains a pore-forming protein termed mitochondrial porin responsible for the exchange of water-soluble metabolites across mitochondrial outer membrane [1–5]. This channel forming protein (called also VDAC) was first reconstituted by Schein et al. [6] from crude extracts of *Paramecium* mitochondria into planar lipid bilayer membranes. Some mitochondrial porins (or VDAC) were studied in detail [1,3,7,8]. The genes of mitochondrial porins are localized in the nuclei of the cells and the protein is synthesized on cytoplasmic ribosomes without N-terminal leader extension [9,10]. The known sequences of mitochondrial porins and that of human porin are not particularly hydrophobic, which means that arrangement of the polypeptide chains in secondary, tertiary, and quaternary structure is presumably responsible for their role as intrinsic membrane proteins [11–13]. Interestingly, porin proteins from mitochondria and Gram-negative bacteria are arranged in β-barrel stave structure in membranes and not in α-helices as channel-forming proteins in nerve and muscle membranes [14]. The direct homology between the known eukaryotic porins of human B-lymphocytes [13], yeast [11] and *Neurospora crassa* [12] is very small although these porins exhibit 100% functional homology [4,15,16].

There exists some evidence that plant mitochondria may also contain one or several porins since Smack and Colombini [17] observed a VDAC in membrane fractions of corn root mitochondria. Furthermore, X-ray diffraction of mitochondrial outer membranes of plants has revealed the presence of porin channels [18]. A 30 kDa molecular mass protein has been isolated from mung bean mitochondria which forms large water-filled channels when reconstituted into vesicles [2]. In this paper we describe reconstitution experiments with detergent extracts from pea mitochondria. A 30 kDa protein is the major channel-forming component of these extracts. It has the characteristics of a slightly

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Abbreviations: BSA, bovine serum albumin; HTP, hydroxyapatite.

anion-selective general diffusion pore with an effective diameter of 1.7 nm in the open state but it is cation-selective in the closed state. This protein is most probably the pea porin. The fractions of a HTP column used for the purification of porin contained also a second membrane channel. Single-channel analysis indicated that the detergent solubilized material formed transient pores with a single-channel conductance of 2.2 nS in 1 M KCl. This channel had also the characteristics of a general diffusion pore with an estimated effective diameter from the single-channel data of about 1.2 nm.

Materials and Methods

Pea growth and isolation of mitochondria. Pea seedlings (*Pisum sativum* var. Kleine Rheinländerin) were grown in greenhouse for 12–16 days on soil. The natural light was supplemented by 150 W incandescent lamps. Pea leave mitochondria were prepared essentially after the method of Neuburger et al. [19] with the exception that 0.3 M sucrose instead of 0.3 M mannitol was used throughout the preparation [20]. The storage medium contained 0.3 M sucrose, 10 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$, 0.1% (weight/vol) defatted BSA (pH 7.2). The integrity of the mitochondria was estimated from the measurement of the consumption of cytochrome *c* and of ascorbate-dependent O_2 according to Neuburger et al. [19]. The integrity of mitochondria was found to be better than 95% in all preparations.

Detergent extracts and purification of pea porin. Detergent extracts of pea mitochondria were prepared essentially based on the porin isolation method described by De Pinto et al. [21]. The mitochondrial pellet (about 15 mg total protein) was suspended in a buffer containing 10 mM Hepes, 1 mM EDTA (pH 6.8), supplemented with 2% Genapol X-80 (Fluka, Neu-Ulm, Germany; final detergent concentration 5 mg Genapol X-80 per mg of protein) and centrifuged for 30 min at $100000 \times g$. The extract was applied to a HTP column preequilibrated and eluted with the same and higher ion strengh buffer in fractions of 1 ml. Pea porin containing fractions were pooled and stored at −25°C.

Black lipid membrane experiments. Methodology for the reconstitution experiments using black lipid bilayer membranes has been previously described [22]. Briefly, the apparatus consisted of a Teflon chamber with a thin wall separating two aqueous compartments. The Teflon divider had small circular holes with an area of either 2 mm^2 (for macroscopic conductance measurements) or 0.1 mm^2 (for single-channel experiments). A 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) in n-decane was painted over the holes to form the membrane. Reconstitution experiments were initiated after the lipid bilayer membrane thinned out and turned optically black to incident light indicating bilayer formation. The aqueous salt solutions (Merck, Darmstadt, Germany) were used unbuffered and had a pH around 6. The temperature was kept at 25°C throughout.

The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and an electrometer (Keithley 602). In the case of the single-channel recordings the electrometer was replaced by a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded with a tape or a strip chart recorder. Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100 to 1000 channels as has been described earlier [23].

Results

Investigation of the mitochondrial pellet for pore-forming activity

The mitochondrial pellet was treated with the detergent Genapol X-80 to solubilize the mitochondrial outer membrane. When the detergent extract was added to the aqueous phase bathing a lipid bilayer membrane a strong pore-forming activity was observed. To identify and separate the pore-forming proteins within the extract, it was applied to a HTP-column which has been previously used for the purification of mitochondrial porins [21]. During chromatography we noticed that a 30 kDa protein did not bind to the column and eluted at high purity, right after the void volume. Purity of the protein was checked with 12% SDS-PAGE (Fig. 1). The apparent molecular mass of the protein was independent of the solubilization tem-

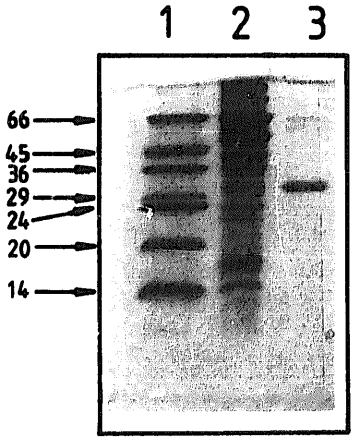

Fig. 1. SDS-PAGE of different purification steps of pea porin. 1, molecular mass markers; 2, supernatant of treatment of the mitochondrial pellet with the Genapol X-80 containing buffer before application to the HTP-column; 3, purified pea porin. The protein migrated on the gel with an apparent molecular mass of about 30 kDa. Coomassie blue staining.

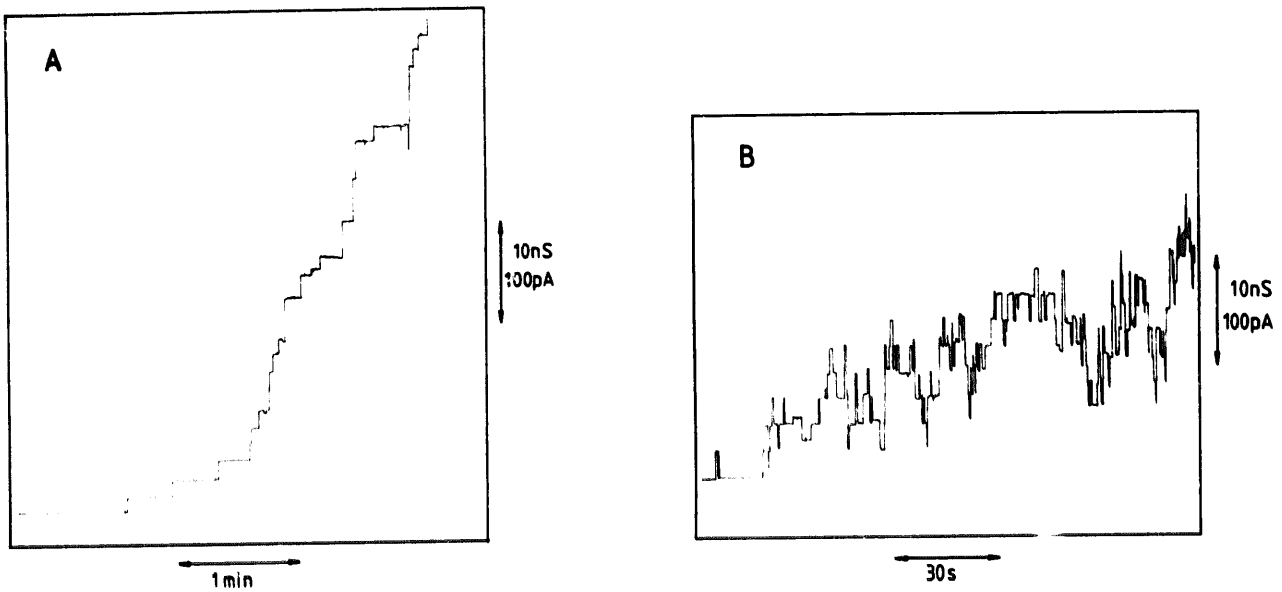

Fig. 2. (A) Stepwise increase of the membrane current (given in pA) after the addition of pea porin to a diphytanoylphosphatidylcholine/n-decane membrane given as a function of time. The aqueous phase contained 5 ng/ml porin and 1 M KCl. The voltage applied was 10 mV; T = 25°C. (B) Single-channel recording of a diphytanoylphosphatidylcholine/n-decane membrane after the addition of 20 μl of fraction 15 from the HTP-column to the aqueous phase. The aqueous phase contained 1 M KCl (pH 6). The applied voltage was 10 mV; the current prior to the addition of fraction 15 was less than 0.5 pA; T = 25°C.

perature and the presence of mercaptoethanol. The 30 kDa protein was identified as a porin by testing the different fractions of the column in lipid bilayer membranes (see below). We noticed also channel-forming activity in fractions which eluted at higher ionic strength. However, we were not able to identify a defined protein in these fractions since the protein concentration was very low in them (10 to 30 μg/ml). Coomassie staining of SDS-PAGE did not reveal any bands. Silver staining showed several faint bands, but it was not possible to correlate the 2.2 nS channel to one of them since fractions with different bands had the same high pore-forming activity.

Single-channel analysis

The detergent extract and the different fractions of the HTP-column were tested in the lipid bilayer system. The addition of small amounts of the extract (final protein concentration 50–100 ng/ml) to the aqueous solution in contact with a lipid bilayer membrane caused a large increase of the specific membrane conductance. Similar effects were observed with frac-

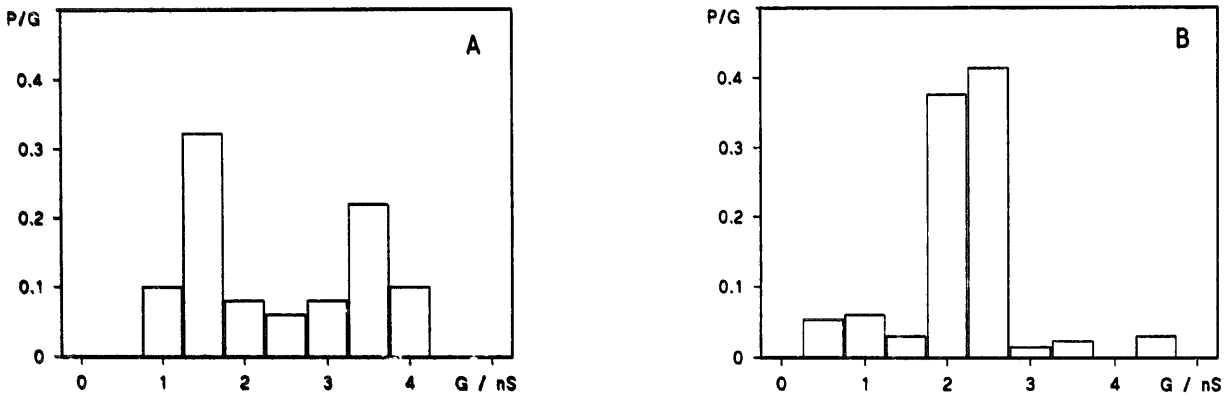

Fig. 3. (A) Histogram of conductance fluctuations observed with membranes of diphytanoylphosphatidylcholine/n-decane in the presence of pea porin. $P(G)$ is the probability for the occurrence of a conductance step with a certain single-channel conductance (given in nS). The aqueous phase contained 1 M KCl. The voltage applied was 10 mV. The mean value of all upward directed steps was 3.7 nS for the right-side maximum and 1.5 nS for the left-side maximum (in total 165 single events); T = 25°C. (B) Histogram of conductance fluctuations observed with membranes of diphytanoylphosphatidylcholine/n-decane in the presence of fraction 15 of the HTP-column. $P(G)$ is the probability for the occurrence of a conductance step with a certain single-channel conductance (given in nS). The aqueous phase contained 1 M KCl. The voltage applied was 10 mV. The mean value of all upward directed steps was 2.2 nS (in total 133 single events); T = 25°C.

tions of the HTP column which eluted just after the void volume of the column (fractions 2 to 4). Only these fractions contained the 30 kDa protein. This protein is the pea porin. Fig. 2A shows a single-channel recording measured with a lipid bilayer membrane in the presence of 5 ng/ml pea porin. The channels had a single-channel conductance of 1.5 and 3.7 nS in 1 M KCl (Fig. 3A). Surprisingly, the histograms obtained with pure protein contained a smaller fraction of the 3.7 nS channel than the crude extracts. Possibly, the purification procedure led to a partial damage of the pore-forming complex.

Lipid bilayer experiments were also performed with other fractions to check them for channel-forming activity. In fractions 10 to 20 we observed a transient channel with a single-channel conductance of 2.2 nS (see Figs. 2B and 3B). As pointed out above it was not possible to identify the protein responsible for the 2.2 nS transient channel since the protein concentration in the fractions was probably too small (10–30 μg/ml) and the channel-forming activity could not be correlated with a defined band in the siver-stained gels. Nevertheless, we would like to point out that the occurrence of the 2.2 nS channel was not a rare event since we observed more than 1000 channels in a membrane although the open and the closed probabilities of the channel are not known. This means that the definitely small protein concentration had probably a high pore-forming activity.

Single-channel conductances of the pea porin and the transient channel were measured in various salt solutions by adding either the 30 kDa pea porin or fractions 10 to 20 of the HTP-column to the aqueous phase. For pea porin the right maximum of the single-channel distribution in 1 M potassium acetate was smaller than that observed in 1 M LiCl (Table I). On the other hand, the single-channel conductance in LiCl was only little smaller than that in KCl. Both results indicated that pea porin was selective for anions. Interestingly, the transient channel was also selective for anions since the mobility of the cation in the aqueous phase had a smaller influence on the single-channel conductance than the anion (see Table I). The single-channel conductance of both channels was a linear function of the bulk aqueous conductance. Such a behavior is expected for wide, water-filled general diffusion channels which contain probably both, positively and negatively charged groups in the vicinity of the channel [5].

Pea porin is voltage-dependent

Single-channel experiments at higher membrane potentials suggested that pea porin is voltage-dependent. The closed state was cation-selective since the conductance of this state was smaller in LiCl than in KCl (data not shown). The voltage-dependence could be demonstrated well in multi-channel experiments as shown in Fig. 4. Voltages of 10, 50 and 100 mV were applied to a membrane in which about 20 pea porin channels were reconstituted. Starting with about 50 mV the current through the channels decreased with time. The ratio of the steady-state conductance divided by the initial conductance showed a bell-shaped curve as a function of voltage. Analysis of the bell-shaped curve by using the same formalism as has been proposed previously [6], suggested that pea porin is less voltage dependent than other mitochondrial porins. The 2.2 nS transient channel was not voltage-dependent until 100 mV.

TABLE I

Average single-channel conductance of pea porin and the transient channel from fractions 10 to 20 of the HTP-column chromatography in different salt solutions of concentration c

The aqueous salt solutions contained either 10 ng/ml porin or about 10–50 μl of the fractions 10 to 20; the pH was between 6.0 and 7.0. The membranes were made from diphytanoylphosphatidylcholine/n-decane; $T = 25°C$; $V_m = 10$ mV. G was determined by recording at least 100 conductance steps and averaging over the distribution of the values. In the case of pea porin only the single-channel data of the right hand maxima of the histograms are given. The single-channel conductances of the left-hand maxima were always approximately 1/2 of those of the right-hand maxima.

Salt	c (M)	G (nS)	
		pea porin	transient channel from fractions 10 to 20
KCl	0.01	0.050	0.022
	0.1	0.42	0.21
	0.3	1.3	0.55
	1	3.7	2.2
	3	10	4.9
NaCl	1	3.3	1.5
LiCl	1	3.2	1.5
KCH_3COO	1	2.6	1.1

Ion selectivity of both channels

We performed zero-current membrane potential measurements with pea porin to study its selectivity in

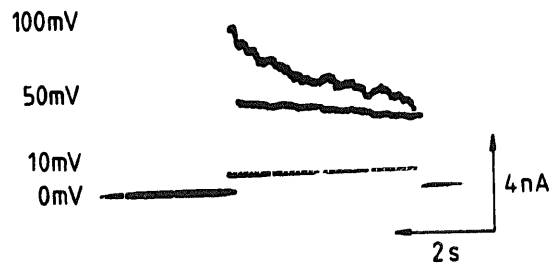

Fig. 4. Relaxation of the membrane current in the presence of 50 ng/ml pea porin added to both sides of the membrane. The membrane potential was switched to 10, 50 and 100 mV. The membrane was formed of diphytanoylphosphatidylcholine/n-decane. The aqueous phase contained 1 M KCl; $T = 25°C$.

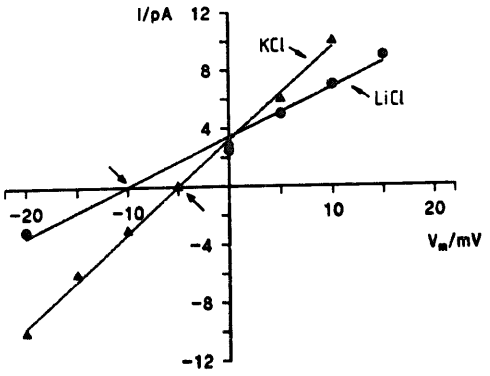

Fig. 5. Reversal potential of the transient channel in KCl (triangles) and in LiCl (crosses). A 2.25-fold gradient (0.45 M versus 0.2 M) was applied to the membranes and the current fluctuations of the transient channel were measured as a function of the externally applied voltage (given with respect to the more dilute side of the membrane); $T = 25°C$. The reversal potential (i.e., the potential at which the current through the channel was zero) was −5 mV and −10 mV for KCl and LiCl, respectively (arrows).

more detail. A 10-fold KCl, LiCl and potassium acetate gradient, across a lipid bilayer membrane in which pea porin was incorporated, resulted in asymmetry potentials of about −7, −20 and 14 mV, respectively, at the more diluted side of the membrane. The zero-current membrane potentials were analysed using the Goldman-Hodgkin-Katz equation [23]. The ratio of the anion permeability P_a divided by the cation permeability P_c was about 1.4, 2.6 and 0.5 for KCl, LiCl and potassium acetate, respectively. These data indicated that the selectivity of pea porin is influenced by the aqueous mobility of the ions in the aqueous phase, i.e., the channel is only anion selective when anions have the same or a higher aqueous mobility than cations.

The selectivity of the transient channel was measured by the determination of its reversal potential in single-channel experiments (i.e., the potential at which the current through the channels was zero). A salt gradient of (0.45 M versus 0.2 M KCl) resulted in a reversal potential of −5 mV (negative at the more dilute side; mean of three experiments) which means that P_a/P_c for KCl was 1.7. The same gradient caused a reversal potential for LiCl of −10 mV (see Fig. 5), which means that P_a/P_c was 2.9. These results were consistent with the single-channel experiments, which already suggested a weak anion selectivity for the transient channel.

Discussion

In this study, we have shown that we are able to reconstitute pore-forming proteins from the mitochondrial membranes of the pea *Pisum sativum*. Two types of channels were observed in lipid bilayer experiments. Channel reconstitution was not a rare event since up to 10000 channels were incorporated in a membrane of 1 mm^2 surface area. Control experiments demonstrated that the formation of both types of channels is not simply an artifact caused by the interaction of detergents with the artificial membranes. One of the channels was formed by a protein with an apparent molecular mass of 30 kDa on SDS-PAGE. Its single-channel conductance was about 1.5 or 3.7 nS in 1 M KCl (left and right hand maxima of the channel distribution). Both maxima represent probably stable states of the same protein since they have been observed in different mitochondrial porin preparations [3–5,8]. On the other hand it has to be noted that the 3.7 nS channel was observed more frequently in crude extracts of mitochondria while the 1.5 nS channel was predominant for the pure 30 kDa protein. This observation may indicate some damage of the pore-forming unit during purification.

The channel conductance of the 30 kDa protein was voltage regulated; transmembrane potentials higher than 20 mV resulted in a reduced conductance. The steady-state conductance, G, divided by G_0, the conductance in the absence of a transmembrane potential, displayed a typical bell-shaped curve (data not shown). The 30 kDa protein is the pea porin since (i) its molecular mass is very close to the molecular masses of mitochondrial porins from other organisms [2–5,8,13, 16], (ii) the single-channel conductance is very similar to that of other mitochondrial porins [1–8,15–17], (iii) the channel conductance is voltage regulated, and (iv) the channel is slightly anion-selective. Furthermore, we recognized in preliminary attempts to sequence pea porin that its N-terminus is blocked which is also typical for mitochondrial porins [11–13]. Pea porin has not been described to date, but it has to be noted that a voltage-dependent channel has been found in crude extracts of corn root mitochondria which is caused probably by the corresponding porin [17].

The molecular mass of the protein responsible for the transient channel is not known at present but a similar channel has also been detected in mitochondria of other organisms especially in those of yeast [24–26]. At present we cannot exclude the possibility that it has a rather low molecular mass or that it is of non-protein in nature (i.e., that it is a polypeptide of low molecular mass). The single-channel conductance of the transient channel is about 2.2 nS in 1 M KCl which suggests that its cross-section could be smaller than that of pea porin. The lifetime of the channel was much shorter than that of pea porin at small voltages. It was on the order of ms to s as Fig. 2B clearly demonstrates.

Both channels are 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

G/σ varied less than a factor of two, i.e., the ions seemed to move inside both pores similar to the way they move in an aqueous environment. This finding allowed a rough estimate of the effective diameter of the pores by assuming a cylindrical shape for them [5,27]. The effective diameter of pea porin and the transient channel were estimated from their average single-channel conductances in 1 M KCl ($G = 3.7$ nS and 2.2 nS, respectively) to be about 1.7 nm and 1.2 nm ($\sigma = 110$ mS/cm). Both diameters would be large enough to allow the passage of solutes of mitochondrial metabolism. It has to be noted that the diameter derived from single-channel experiments has to be taken as a lower limit of channel size since the lipid bilayer technique tends to underestimate the channel size under certain conditions. In particular, it may be caused by the decrease of the maximal possible channel conductance (according to the dimensions of the channel) due to incomplete shielding of the channel interior against the low dielectric membrane interior.

Only pea porin may normally be responsible for the exchange of mitochondrial metabolites across the mitochondrial outer membrane since porin inhibitors block the exchange of ATP and ADP across this membrane [30]. The function of the transient channel in pea mitochondria is not known, although a similar channel in mitochondrial outer membrane of yeast serves as an 'emergency' channel for ATP, ADP and other mitochondrial metabolites when yeast porin is deleted [28,29]. Nevertheless, the yeast mutant shows a reduced respiration activity under normal conditions [28,29]. This suggests that the passage of mitochondrial metabolites may not be its 'normal' function. Interestingly, in a recent paper it has been suggested that the transient channel of yeast has to do with the protein transport (i.e., protein import into mitochondria) across the outer membrane since it was blocked by the addition of signal peptides [25]. The transient channel investigated here is in contrast to the corresponding channel of yeast mitochondria anion-selective. If it is involved in protein translocation across mitochondrial outer membrane of pea mitochondria is an open question.

In previous publications [30,31] 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 [32]. Rat liver porin becomes impermeable for ATP and ADP in the presence of this inhibitor because the adenylate kinase activity could be blocked in a reversible way [30]. These facts provided clear evidence that the mitochondrial metabolism could be controled by the outer membrane pore. Similar investigations have not been performed with plant mitochondria but there exists little doubt that the inhibitor has a similar effect there. This indicates that the function of the mitochondrial outer membrane is still not fully understood at present and deserves further investigations.

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