Multi-author Review

Pore-forming proteins of biological membranes

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Introduction

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Biological membranes which separate different intra- and extracellular compartments from each other are necessarily barriers for a large number of substances. Apart from hydrophobic molecules only water and gases like CO_2, O_2 and N_2 permeate phospholipid bilayers by simple diffusion. Since most biological membranes (e.g. the plasma membrane, the inner membrane of mitochondria and bacteria, the ER membrane) have evolved to maintain ion gradients between different compartments, highly specific transport mechanisms are necessary which will allow single molecules selectively to cross the membrane. Specificity is achieved with the aid of a transport protein which 'recognizes' only one substance and either helps its diffusion through the membrane (= *passive transport*, facilitated diffusion), or actively 'pumps' the molecule into another compartment with the consumption of ATP, or transports one molecule at the expense of another (= *active transport* through ATPases or symport/antiport) in which case a gradient can be established.

The outer membranes of bacteria and of mitochondria are exceptional in that they need to maintain neither a gradient nor an electrochemical potential. In fact, their permeability to a variety of hydrophilic substances makes them appear like molecular sieves. The actual 'holes' in the outer membrane are formed by channel proteins called 'porins' in both prokaryotes and mitochondria. They are analogous in their function, but do not share any sequence homology. Since these pores are large and water-filled, hydrophilic substances are thought to permeate through them by simple diffusion. Equally non-specific but completely different in their structure and mechanism of action are killer-pores which are known as 'colicins' in prokaryotes and 'killer-toxin' of virus-like particles in eukaryotes (yeasts). In both cases a secreted protein becomes toxic by being inserted into a foreign membrane and destroying its potential via channel-formation.

How do all these pores work in detail? How are they synthesized and what is their importance in vivo? What are the features common to both prokaryotic and eukaryotic pores? The following reviews attempt to give up-to-date answers to these questions in discussing the structure, function and biogenesis of different eukaryotic and prokaryotic non-specific pore proteins.

Biophysical properties of porin pores from mitochondrial outer membrane of eukaryotic cells

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Summary. The matrix space of mitochondria is surrounded by two membranes. The mitochondrial inner membrane contains the respiration chain and a large number of highly specific carriers for the mostly anionic substrates of mitochondrial metabolism. In contrast to this the permeability properties of the mitochondrial outer membrane are by far less specific. It acts as a molecular sieve for hydrophilic molecules with a defined exclusion limit around 3000 Da. Responsible for the extremely high permeability of the mitochondrial outer membrane is the presence of a pore-forming protein termed mitochondrial porin. Mitochondrial porins have been isolated from a variety of eukaryotic cells. They are basic proteins with molecular masses between 30 and 35 kDa. Reconstitution experiments define their function as pore-forming components with a single-channel conductance of about 0.40 nS (nano Siemens) in 0.1 M KCl at low voltages. In the open state mitochondrial porin behaves as a general diffusion pore with an effective diameter of 1.7 nm. Eukaryotic porins are slightly anion-selective in the open state but become cation-selective after voltage-dependent closure.

Key words. Mitochondria; outer membrane; voltage-dependence; single-channel conductance; lipid bilayer membrane; reconstitution.

Introduction

The outer membrane of mitochondria seems to be freely permeable to a variety of different solutes[33]. It is, on the other hand, impermeable to molecules with higher molecular weights[33]. This indicated a defined pathway in the outer membrane for hydrophilic solutes. In fact, a channel has been identified in the outer mitochondrial membrane of a variety of eukaryotic cells [1,9,10,12,16,21,23,25,31,34]. This channel was named VDAC[9,32] or mitochondrial porin in analogy to the bacterial porins and in agreement with the endosymbiontic theory[1,5,16,21,31,34]. In fact, both mitochondrial and bacterial porins share some similarities, although no sequence homology has been found. Their amino acid composition exhibits a high polarity despite the fact that the channel forming unit contains many membrane spanning segments[2,18,29]. The mitochondrial porins are arranged in β-pleated sheet structure with only little indication for α-helices[18,29] (see also the article by Dihanich, this issue, for the biogenesis of mitochondrial porins). Mitochondrial porins have basic pI's and molecular masses between 30 000 and 35 000 Da[12,21,31,34]. They form in reconstituted vesicles pores with exclusion limits around 3000 Da[10,21].

This short review is focussed on the biophysical characterization of the mitochondrial pore reconstituted into lipid bilayer membranes. These experiments yield detailed information about the pore interior and pore selectivity. Furthermore, lipid bilayer experiments provide an easy way for the study of the voltage dependence of a membrane channel like the mitochondrial porin. The results suggest that the mitochondrial porins form large, weakly anion-selective water-filled channels with effective diameters around 2 nm in the 'open' state[1,5,12]. As the consequence of membrane potentials larger than 20–30 mV they switch to 'closed' states of reduced permeability and completely different selectivity. In particular, adenosin triphosphate (ATP) and -diphosphate are not permeable through the closed channel[6] (see also the contribution by Brdiczka; this issue). The possible role of this voltage dependence is discussed below. The role of the mitochondrial outer membrane in physiology and metabolism of the cell organelle has been underestimated in the past. More recent papers give some insight into the function of the mitochondrial outer membrane and its role in mitochondrial metabolism[6,8,17,30].

Reconstitution of mitochondrial porin into lipid bilayer membranes

Isolation and purification of mitochondrial porins

Mitochondrial porins can be isolated from outer mitochondrial membranes and whole mitochondria following standard procedures. In the first case porin is obtained from Triton-solubilized outer membranes by successive chromatography over a DEAE-Sepharose column and a CM-Sepharose column[21]. Porin is not absorbed to both column materials and appears shortly after the void volume of the column. The yield of this method is relatively low because a large amount of porin is lost during the preparation of the mitochondrial outer membranes. More efficient is the method that starts from whole mitochondria and uses a hydroxyapatite column as an essential step[12,13]. The cell organelles are extracted with the neutral detergents Genapol X-80 or Triton X-100. Then the extract is applied to dry hydroxyapatite (HTP) and appears in the pass-through of the column because porin is deeply buried in the detergent micelle and is therefore not absorbed to the HTP-column[12,19]. Mitochondrial porin obtained by both methods shows pore-forming activity in lipid vesicles and lipid bilayer membranes[12,13,21]. The use of ionic detergents such as sodium dodecylsulfate (SDS) instead of Triton or Genapol dissociates the pore-forming unit and destroys the pore formation in reconstituted systems.

Reconstitution method

The reconstitution of mitochondrial porin is performed as follows[9,16,31]: Purified porin is added in small concentration (10 ng/ml to 1 µg/ml) to the aqueous phase bathing a black lipid bilayer membrane formed according to different methods. Subsequently, the membrane current starts to increase after an initial lag of about two min by many orders of magnitude within 15–20 min[1,5]. This process indicates the insertion of membrane-active ion-permeable material into the membrane. After that time, the membrane conductance (i.e. the current per unit voltage) increased at a much slower rate. This slow conductance increase continued usually until membrane breakage. When the rate of conductance increase was relatively slow (as compared with the initial one) it could be shown for different mitochondrial porins that the membrane conductance was a linear function of the protein concentration up to porin concentrations of about 1 µg/ml[16,24,31]. This result is consistent with the assumption that the protein samples contained a preformed pore.

Characterization of the pore-forming properties

Single-channel analysis and pore diameter

The addition of smaller amounts of mitochondrial porin from different eukaryotic cells allowed the resolution of step increases in conductance as shown in figure 1 for yeast porin. This means that the membrane activity described above is caused by the formation of ion-permeable channels in the membranes and one step reflects the insertion of one conductive unit (i.e. of one channel into the membrane). These conductance steps were specific to the presence of the porins and were not observed when only the detergents Triton X-100 or Genapol X-80 were added to the aqueous phase. Most of the conductance steps were directed upwards and closing steps were only

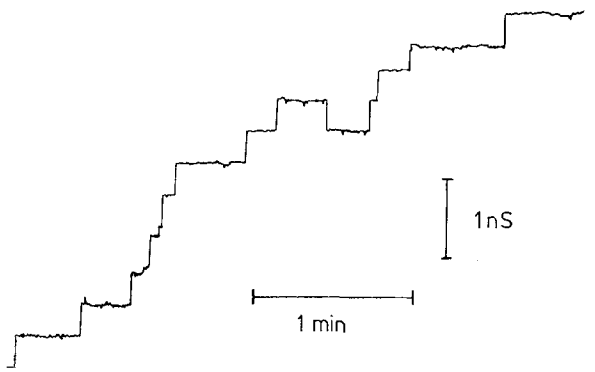

Figure 1. Single channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane after the addition of 5 ng/ml yeast porin to the aqueous phase. The aqueous phase contained 0.1 M KCl, pH 6. The applied voltage was 10 mV; T = 25 °C.

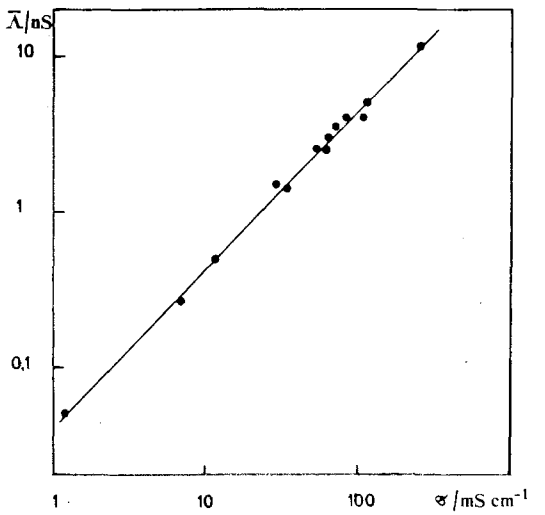

Figure 3. Average single-channel conductance, Λ, of rat brain porin in different salts given as a function of the specific conductivity of the corresponding aqueous salt solutions. T = 25 °C. The results were taken from Ludwig et al.[24] (with permission).

rarely observed at small transmembrane potentials of about 10 mV (fig. 1). The most frequent value for the single-channel conductance of yeast porin in 0.1 M KCl (the conditions of fig. 1) was about 0.4 nS. Only a limited number of smaller steps were observed (see fig. 2 for a histogram of the conductance fluctuations). It should be noted that smaller steps were also found for porins from *Neurospora crassa*[15], rat liver[31], and a number of other cells from mammalian tissues[12]. They have been explained as substates of the pore[5,16]. However, in lipid bilayer experiments in the presence of porin from *Paramecium* the small pores with a single channel conductance around 0.25 nS were exclusively observed in 0.1 M KCl[23].

This result indicated that the mitochondrial pore could not only switch to substates but that it may also exist in two different stable conformations.

A large variety of different ions were found to be permeable through the open state of mitochondrial porins. Furthermore, it has been found that the single-channel conductance of mitochondrial porins is a linear function of the specific conductance, σ, of the bulk aqueous phase at small membrane potentials. This is shown in figure 3 for porin from rat brain mitochondria. Figure 3 indicates that despite a large variation of the average single channel conductance as a function of the bulk aqueous conductivity the ratio Λ/σ varied only little[24,31].

The single-channel conductance, Λ, of the open state can be used to calculate the effective diameter of the pores according to an established procedure[1]. Assuming that the pores are filled with a solution of the same specific conductivity, σ, as the bulk aqueous solution and assuming a cylindrical pore with a length l of 6 nm (corresponding to the thickness of the outer membrane), the effective pore diameter d (= 2r) can be calculated according to the equation[3]:

$$\Lambda = \sigma \pi r^2/l \qquad (1)$$

Table 1 contains the effective diameters of a number of porin pores from different eukaryotic cells calculated according to eqn. (1) from the conductance of the pores in 1 M KCl (σ = 110 mS/cm). The effective diameter of most pores is about 1.7 nm, whereas that of the *Paramecium* porin is only 1.3 nm[12,16,23,25,31]. Such a diameter is consistent with the results obtained with isolated mitochondria[33] and by electron microscopy of mitochondrial

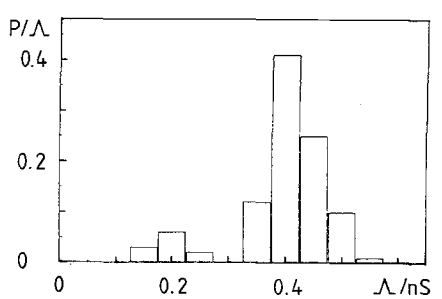

Figure 2. Histogram of conductance fluctuations observed with membranes of diphytanoyl phosphatidylcholine/n-decane in the presence of yeast porin. The aqueous phase contained 0.1 M KCl. The applied voltage was 10 mV. The mean value of all conductance steps was 0.42 nS for 159 single events. V_m = 10 mV; T = 25 °C.

Table 1. Single channel conductances of porins from different eukaryotic cells in 1 M KCl.

Porin	Λ (nS)	d (nm)	Ref.
Pig heart	3.5	1.6	12
Rat liver	4.3	1.7	1, 31
Rat brain	4.0	1.7	24
Neurospora crassa	4.5	1.8	16
Yeast	4.2	1.7	25
Paramecium	2.4	1.3	23

The effective diameter d of the pores was calculated assuming the length of the pore to be 6 nm and a specific conductance σ of the pore interior of 110 mS/cm. The membranes were made of diphytanoyl phosphatidylcholine/n-decane. The voltage was 10 mV; T = 25 °C.

outer membranes[26,27] which suggest a diameter between 2 and 2.5 nm. It disagrees, however, with the diameter of 4 nm obtained by Colombini[10] for the diffusion of polyethylene glycols through the mitochondrial pore. The reason for this discrepancy is not clear. It may come from the possibility of single-file movement of the polyethylene glycols through the channel.

Ion selectivity of mitochondrial porins

Ions move through the open state of mitochondrial porin similar to the way they move in bulk aqueous phase[2]. Nevertheless, the pores exhibit a certain specificity for charged solutes, i.e. the channel has a limited selectivity for ions[1,5,16,31]. The lipid bilayer assay allows the evaluation of the ionic selectivity by measuring the membrane potential under zero-current conditions. From the measured V_m as a consequence of the externally applied concentration gradient, c"/c', across the membrane, the ratio P_c/P_a of the permeabilities (P_c for cations and P_a for anions) can be calculated using the Goldman-Hodgkin-Katz equation[4]. Table 2 shows the zero-current membrane potentials and the permeability ratios for mitochondrial porins of *Paramecium* and yeast in potassium chloride, potassium acetate and lithium chloride. It is obvious from the data of table 2, that the asymmetry potential and the ion selectivity of mitochondrial porin is dependent on the combination of different anions and cations. The channel is slightly anion-selective for the equally mobile potassium and chloride ions. For the combination of the less mobile lithium ion (because of the larger hydration shell) with chloride the anion selectivity increases. Surprisingly, the channel becomes cation-selective for potassium acetate presumably because of the small mobility of acetate as compared with chloride. This means that the ions move inside the pore similar to the way they move in the aqueous phase and support the assumption of the mitochondrial pore in the 'open' state as a wide water- filled channel.

Table 2. Zero-current membrane potentials, V_m, of membranes from diphytanoyl phosphatidylcholine/n-decane in the presence of *Paramecium*[23] and yeast[25] porin measured for a 10-fold gradient of different salts*.

Salt	V_m (mV)	P_a/P_c
	Paramecium	
KCl (pH 6)	−11	1.7
LiCl (pH 6)	−24	3.4
Potassium acetate (pH 7)	+14	0.5
	Yeast	
KCl (pH 6)	−7	1.4
LiCl (pH 6)	−20	2.6
Potassium acetate (pH 7)	+14	0.5

* V_m is defined as the potential of the dilute side (10 mM) relative to that of the concentrated side (100 mM); the temperature was 25 °C. P_a/P_c was calculated from the Goldman-Hodgkin-Katz equation[4] from at least 4 individual experiments.

Mitochondrial porin is voltage-dependent

Evaluation of the voltage dependence

At 10 mV transmembrane potential, the terminating events in single channel recordings represented only a minor fraction of the current fluctuations. At higher voltages, beginning with about 20–25 mV, the terminating events became more and more frequent. They were often smaller than the initial on-steps. This result indicates that the pore switches to substates at high voltages. The closed states still allow the permeation of ions through the pore. This is shown in figure 4. Transmembrane potentials of 35 and −35 mV were applied to a membrane from yeast containing many mitochondrial pores (5×10^5 pores/cm^2). The membrane conductance decreases in a somewhat asymmetric fashion. The voltage dependence of the mitochondrial porins can be demonstrated in experiments in which only a few channels were reconstituted into the membrane and in multi-channel experiments. In the multi-channel system the decay of the membrane current following a voltage step could be described by a single exponential decay (fig. 4). The steady-state conductance showed a bell-shaped curve as a function of the applied voltage as shown for yeast porin in figure 5. The data given in figure 5 were analyzed as proposed by Schein et al.[32]:

$$N_o/N_c = \exp\,(nF\,(V_m - V_0)/RT) \qquad (2)$$

where F, R and T have the usual meaning, n is the number of gating charges moving through the entire transmembrane potential gradient for channel gating and V_0 is the potential at which 50% of the total number of channels are in the closed configuration. The open to closed ratio of the channels, N_o/N_c, may be calculated from the data given above according to:

$$N_o/N_c = (G - G_{min})/(G_0 - G) \qquad (3)$$

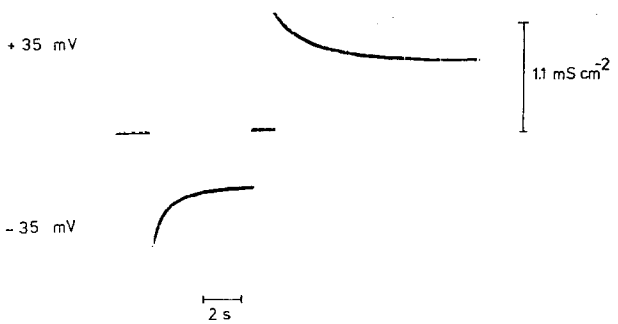

Figure 4. Voltage dependence of yeast porin. About 5×10^5 porin pores per cm^2 were incorporated in a membrane from 1% diphytanoyl phosphatidylcholine/n-decane. The voltage across the membrane was switched to −35 mV (with respect to the cis-side, the side of the addition of 500 ng/ml protein) and then to 35 mV. The channels switched to substates of the open state. The aqueous phase contained 1 M KCl; T = 25 °C.

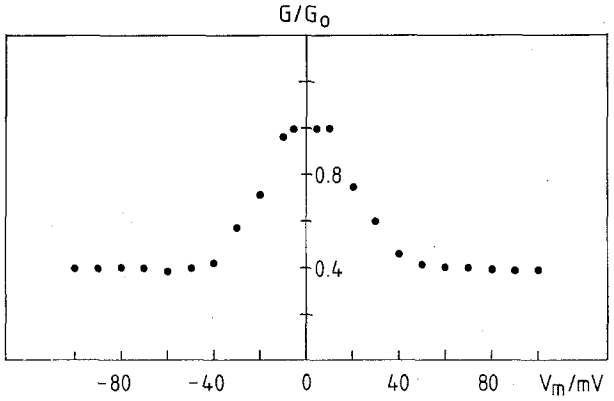

Figure 5. Ratio of the conductance G at a given membrane potential divided by the conductance G_0 at zero potential as a function of the applied membrane potential V_m. The membranes were formed of diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 M KCl. The cis-side contained 20 ng/ml yeast porin. The voltage is given with respect to the cis-side; T = 25 °C.

G in this equation is the conductance at a given membrane potential V_m, G_0 and G_{min} are the conductances at zero voltage and very high potentials, respectively. A semilogarithmic plot of the data given in figure 5 showed that they could be fitted to a straight line with a slope of 13 mV for an e-fold change of V_m. This result suggested that the number of charges involved in the gating process was approximately two in the case of yeast porin (see fig. 6). The voltage dependence of the mitochondrial porins varies for different sources. Whereas 35 mV were sufficient to decrease the initial conductance of yeast porin to 50%, about 90 mV had to be applied to membranes containing porin from rat brain to obtain the same effect [24]. This result indicated that the number of charges involved in the gating process varies for mitochondrial porins from different organisms.

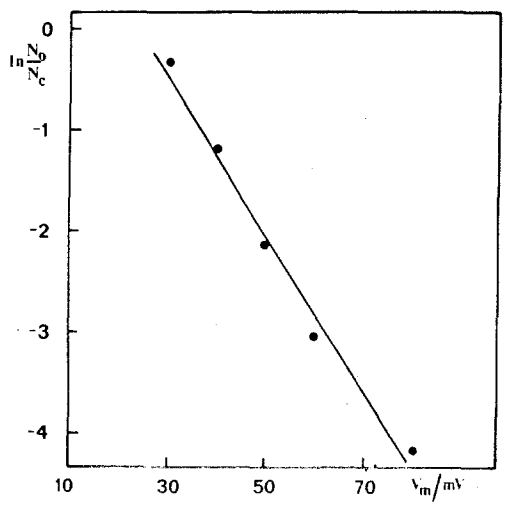

Figure 6. Semilogarithmic plot of the ratio N_o/N_c as a function of the transmembrane potential V_m. The data were calculated from the results of fig. 5 using eqns (2) and (3). The slope of the line was about 13 mV for an e-fold change of N_o/N_c; V_0 = 16 mV. Only the data of the positive branch of fig. 5 is shown.

Table 3. Average single channel conductance of the open and closed state of yeast porin in different 0.5 M salt solutions. 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.

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

Ionic selectivity of the closed state

The open state of all mitochondrial porins characterized so far is slightly anion-selective for salts with equally mobile cations and anions such as KCl (see above). This means that the ionic selectivity is dependent on the mobility of the ions in the aqueous phase. Such a behavior is expected for a general diffusion pore because ions move through this pathway similar to the way they move through the aqueous phase. The zero-current membrane potentials were of the order of −10 mV at the more dilute side of a tenfold KCl gradient [1, 5, 12] which corresponds to a twofold higher permeability for Cl^- over K^+. As described above, the mitochondrial porins switched to closed states when the transmembrane voltages exceeded 15–20 mV. These states definitely have a reduced permeability towards ions. Their ionic selectivity is difficult to measure, because it is impossible to perform zero-current membrane potential measurements under the conditions in which the pores are in the closed state. To get some insight into the ionic selectivity of the closed state single channel experiments were performed in salts combined of anions and cations of different mobility. 30 mV transmembrane potential was applied to the membranes and yeast porin was added in a small concentration. Because of the voltage dependence the open state of yeast porin had only a limited lifetime under these conditions which means that it was possible to measure the single channel conductance of the closed state by subtracting the conductance of the closing events from those of the open state. Table 3 shows the results of this type of measurement obtained for three different salts. The single channel conductance of the closed state of the pore was considerably smaller for Tris-HCl 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.

Inhibition of the mitochondrial pore

König and coworkers [19, 20] have shown that a synthetic polyanion (a copolymer of M_r 10,000 of methacrylate, maleate and styrene in a 1:2:3 proportion) inhibits transport across the mitochondrial inner membrane. It is now clear that this inhibition is not caused by a direct interaction between polyanion and inner membrane carriers. It seems moreover, that polyanion binds to mitochondrial

porin and shifts its voltage dependence in a oriented way[6,11]. Small membrane potentials, negative at the cis-side (the side of the addition of the polyanion) are able to close the channel and inhibit the passage of anionic metabolites across the mitochondrial outer membrane thus blocking mitochondrial metabolism[6]. Other polyanions have a similar effect on the voltage dependence of mitochondrial porin[11].

Conclusion

Mitochondrial porins obviously form large water-filled channels in lipid bilayer membranes in the 'open' state because large organic ions can enter the pores without much interaction with the pore interior. Furthermore, the pore conductance shows no saturation with increasing salt concentration which would in principle be expected for an ion-specific pore. The 'open' state of most mitochondrial porins has effective diameters of about 1.7 nm. Only porin from *Paramecium* has a smaller effective cross section. So far it is not clear if a basic difference exists between this porin pore and the others because single channel conductances of 0.25 nS in 0.1 M KCl have also been observed for other mitochondrial porins. This could mean that mitochondrial porins could exist in different stable configurations with different cross sections for pores in the open state.

The origin of mitochondria is still puzzling. The endosymbiotic theory postulates that the symbiosis of an advanced protoeukaryote with one strictly aerobic gram-negative prokaryote led to the first eukaryotic cell. Mitochondria and the plastids have, in agreement with this hypothesis, an outer membrane, a circular DNA, and they divide in a similar way to bacteria. Furthermore, the respiration chain and the photosynthetic apparatus have evolved only once. The outer membranes of the cell organelles contain permeability channels whose structure and function are similar to those in the outer membrane of gram-negative bacteria, although no sequence homology has been shown to date[1,2].

The basic difference between bacterial and mitochondrial porins is the voltage-dependence of the latter.

Mitochondrial porins form voltage-dependent pores in lipid bilayer membranes. The single channel conductance switches to substates as a result of increasing voltage. The lower conductance states of the mitochondrial pore have a different permeability and selectivity than the open pores. In the case of yeast and *Paramecium* porin, the lower conductance state is cation-selective[23,25]. This result is consistent with the assumption that the permeability of the mitochondrial outer membrane is voltage-controlled. Increasing transmembrane potentials reduce the exclusion limit and change the selectivity of the outer membrane and thus control mitochondrial metabolism. In fact, evidence has been found for such a regulation process because König's polyanion (a copolymer of M_r 10,000 of methacrylate, maleate and styrene in a 1:2:3 proportion)[19], known as a potent inhibitor of mitochondrial metabolism[19,20], forces the pore into the closed state. In this state the pore is impermeable for ATP and ADP[6]. The voltage across the outer membrane could be generated by capacitive coupling between inner and outer membrane because of their small distance in *in situ* mitochondria[1]. Further support for the important role of mitochondrial porin arises from the study of porin-free yeast mutants[14,28].

The mitochondrial pore presumably also plays an important role in the formation of different compartments in mitochondria (see the contribution by Brdiczka, this issue). The basis for the formation of compartments is presumably the binding of hexokinase and glycerokinase to the pore, i.e. the mitochondrial porin is identical to the hexokinase binding protein[8,15,22].

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Structural analysis of mitochondrial pores

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Summary. Structural information about the channel in the mitochondrial outer membrane, derived from sequence analysis and electron microscopy of two-dimensional crystals, is summarized. A model for the channel is presented, consisting of a cylindrical beta-barrel that is formed by one or two 30-kDa polypeptides, with an alpha-carbon backbone diameter of 3.8 nm. The radial distributions of basic amino acids and lipid-contact regions on the projected cylinder are mapped relative to interchannel bonding sites inferred from channel packing in the arrays. Speculation on the kinds of conformational changes that the channel might undergo is also presented.
Key words. Mitochondrial outer membrane; channels; membrane crystals; electron microscopy; image processing.

Voltage-gated ion channels play a central role in several biological processes. Yet, it is not an overstatement to say that we are generally ignorant of the molecular mechanisms underlying the operation of these channels. Basic unanswered questions include: How may polypeptides fold to make a polar, transmembrane compartment? What are the physical parameters that control the selectivity of channels? And what kinds of conformational changes are involved in gating? Answering these questions will require detailed understanding of the structure and dynamics of these intriguing cellular components.

Much of our current conception of the higher order structure of gated channels comes from two sources: polypeptide folding schemes inferred from sequence analysis[15], and low-resolution models of the channels in gap junctions and acetylcholine receptors derived from X-ray scattering[23] and electron microscopic data[4,54]. The picture taking shape is one of homologous protein domains (four to six, contributed by one or more polypeptides) interacting laterally in the membrane to form one transverse polar compartment. The predominant structural elements are bundles of alpha-helices aligned normal to the membrane, twenty or more coming together to form a single pore. The total protein mass involved in making one of these channels is generally 200 kDa or greater.