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Porin pores of mitochondrial outer membranes from high and low eukaryotic cells: biochemical and biophysical characterization

Vito De Pinto [a], Otto Ludwig [b], Jürgen Krause [c], Roland Benz [b] and Ferdinando Palmieri [a]

[a] *Department of Pharmaco-Biology, Laboratory of Biochemistry, University of Bari and CNR Unit for the Study of Mitochondria, Bari (Italy),* [b] *Lehrstuhl für Biotechnologie, Universität Würzburg, Würzburg and* [c] *Fakultät für Biologie, Universität Konstanz, Konstanz (F.R.G.)*

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The mitochondrial porins from mammalian tissues and from low eukaryotic cells were purified with a high yield, and their biochemical and functional properties were investigated. When analyzed by SDS gel electrophoresis, all mammalian porins show a very similar apparent molecular mass (35–35.5 kDa). In contrast yeast and *Paramecium* porins have a molecular mass of 30 and 37 kDa, respectively. The peptide maps of mammalian porins are very similar although small differences are apparent between porins of different tissues of the same organism and also between those of the same tissue of different organisms. The peptide patterns of porins from yeast and *Paramecium* are completely different from those of mammalian porins. Antibodies raised against the rat liver porin cross-react with all the other mammalian porins but not with that of yeast. The incorporation of porins into artificial lipid bilayer membranes showed that they are able to form pores with approximately the same specific activity. The single-channel conductance is for all porins, except for that of *Paramecium*, about 4 nS in 1 M KCl, corresponding to an effective pore diameter of 1.7 nm. They are voltage-dependent and switch to substates at transmembrane potentials higher than 10 mV. The number of gating charges varies, however, for pores from different tissues, indicating a different sensitivity to the potential as a result of a possible different function.

Introduction

The matrix space of mitochondria is surrounded by two unit membranes. Whereas the role of the inner membrane in oxidative phosphorylation was studied in full detail in recent years, the role of the mitochondrial outer membrane in the mitochondrial metabolism has been neglected because of its high permeability for small hydrophilic solutes [1,2]. More recently an important function of the outer mitochondrial membrane in the energy conversion of mitochondria and the import of proteins from the cytosol was established [3,4]. Furthermore, at least one pore-forming protein, the mitochondrial porin or voltage-dependent anion channel, was identified in the mitochondrial outer membrane [5–10]. The pores formed by the mitochondrial porin share some similarities with the bacterial porins located in the bacterial outer membranes (see Ref. 11 for a recent review).

Abbreviations: DCCD, dicyclohexylcarbodiimide.

Correspondence: F. Palmieri, Department of Pharmaco-Biology, Laboratory of Biochemistry, Via Amendola 165/A, I-70126 Bari, Italy.

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Some properties and the biosynthesis of mitochondrial porins from *Neurospora crassa* and yeast were studied in detail. It could be shown that the gene of the mitochondrial porin is located in the nucleus of the cell and the protein is synthesized at cytoplasmic ribosomes without leader sequence [12–14]. The primary sequence of the mitochondrial porin from yeast was derived from the sequence of the gene [15]. The primary sequence is not particularly hydrophobic. This result indicated that the arrangement of the polypeptide chain in secondary, tertiary and quaternary structure is responsible for its role as an intrinsic membrane protein [15]. Furthermore, it is most likely that the pore-forming complex is composed of one or several polypeptide chain(s), lipid, and sterol [16,17].

In comparison with the knowledges available on the *N. crassa* and yeast porins, only little is known about the structure and the possible function of the pore-forming protein of the mitochondrial outer membrane of mammalian cells. In this paper we have isolated and purified several mitochondrial porins from different mammalian tissues and from low eukaryotic cells. The porins were characterized with respect to their molecular weight, to their peptide maps, and to their immunological cross-reaction with antibodies. Reconstitution experiments with lipid bilayer membranes showed that all mammalian and low eukariotic porins so far isolated formed large general diffusion pores. The single-channel conductance, the ionic selectivity, the voltage dependence and the parameters of the gating process have been determined.

Materials and Methods

Materials. Affi-Gel 501 and hydroxyapatite (Bio-gel HTP) were obtained from Bio-Rad, Triton X-100, acrylamide and *N,N'*-methylenebisacrylamide from Serva. Celite 535 was purchased from Roth, *Staphylococcus aureus* V 8 proteinase from Miles laboratories, papain from Sigma and carbonic anhydrase from Boehringer Biochemia. All the other reagents were of the highest purity commercially available.

Purification of mitochondrial porins. Mitochondria from different organs and species of mammalian were purified by standard procedures. The isolation of the various mammalian porins was performed essentially as previously described for the purification of the 35 kDa DCCD binding protein [18]. 80 mg of mitochondria were solubilized with 8 ml 3% Triton X-100, 20 mM KH_2PO_4 (pH 6.5), 20 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA). After 20 min at 0 °C, the mixture was centrifuged at 147 000 × *g* for 30 min. The Triton X-100 extract (i.e., the clear supernatant) was applied to a dry hydroxyapatite column (6 g of dry material) eluted with the solubilization buffer. The first 7 ml of the hydroxyapatite eluate were applied to an Affi-Gel 501 column, preequilibrated with the solubilization buffer. The unretarded proteins from the Affi-Gel column were applied to a dry hydroxyapatite/celite column (6 g of dry material; ratio 1 : 1, w/w) and eluted with the solubilization buffer. The first 6 ml of the eluate from the hydroxyapatite/celite column were collected and analyzed. The porin from yeast was purified as in Ref. 19 and that from *Paramecium* as in Ref. 20.

SDS gel electrophoresis. Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [21]. The separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150. In order to determine the content of porin in whole mitochondria, these were directly dissolved in SDS and subjected to electrophoresis on gels containing a linear gradient of acrylamide from 12 to 18% and an acrylamide/bisacrylamide ratio of 30 : 0.8. Gels were stained with Coomassie blue or with silver [22].

Peptide mapping. The proteolysis was performed on slices of SDS gel or in vitro on purified proteins according to Cleveland [23]. In the first case, purified porins were subjected to electrophoresis on a standard gel 0.75 mm thick. After Coomassie blue staining, the protein bands corresponding to porins were cut out and equilibrated with 0.125 M Tris-HCl (pH 6.8), 10% glycerol, 0.3% 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% SDS for 30 min. They were then pushed to the bottom of the wells of a 1.5 mm thick gel and covered with the same buffer containing 20% glycerol, on top of which the solution of proteinase was overlayered. The

separating gel was 9 cm high and contained 16% acrylamide (acrylamide/bisacrylamide = 30 : 0.8). The stacking gel was 4 cm high. The electrophoretic run was very slow in the stacking gel (i.e., 4 mA current constant). When the samples had stacked, the power was turned off for 30 min. Finally the rate of migration was increased in the separating gel (i.e., up to 25 mA current constant).

Proteolysis in vitro was performed on purified proteins after precipitation in 90% cold acetone. The pellets were dissolved in SDS sample buffer [21] and proteolytic digestion was carried out at 37 °C. The reaction was stopped by 10% 2-mercaptoethanol and 2% SDS and by boiling for 2 min. The gel electrophoresis was performed with a separating gel containing 16% acrylamide.

Antibody preparation. Porin purified from outer mitochondrial membrane of 40–50 rats (about 1–2 mg) was used. The protein was precipitated from 1% Triton X-100 solution with acetone, 500 μg of the precipitate were dissolved in 2 ml of 0.9% sodium chloride solution, mixed with 2 ml of Freund's complete adjuvant and injected in several portions in a rabbit. 200 μg of porin were again injected after 3 weeks. This procedure was repeated after another two weeks. Blood was taken from rabbit ear. The antibodies were precipitated by 16% Na_2SO_4 solution and were partially purified.

Electrotransfer and immunodecoration. The proteins separated on 10% acrylamide slab gels were transferred to nitrocellulose [24,25]. The transferred proteins were stained with Ponceau-S solution (Serva, F.R.G.), destained, incubated with antibodies raised against the porin purified from rat outer mitochondrial membrane and then incubated with peroxidase-conjugated protein A. The peroxidase reaction was performed in a mixture of 9 ml 4-chloro-1-naphthol (0.3% in methanol), 141 ml 0.14 M NaCl buffered with 10 mM phosphate (pH 7.0) and 60 μl 35% H_2O_2.

Membrane experiments. The methods used for black lipid bilayer experiments have been described previously [26]. The apparatus consisted of a Teflon chamber with two aqueous compartments. Circular holes in the wall between the two compartments had an area of either 2 mm^2 (for macroscopic conductance measurements) or about 0.1 mm^2 (for single-channel experiments). Membranes were formed across the holes by painting on a 1% (w/v) solution of a 4:1 mixture of diphytanoylphosphatidylcholine and phosphatidylserine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The aqueous solutions were used unbuffered with a pH of 6. To prevent protein inactivation, the protein was added to the aqueous phase from the 10-fold diluted stock solution (containing 0.1% Triton X-100) either prior to membrane formation or after the membrane had turned completely black. The temperature was kept at 25 °C throughout.

The membrane current was measured at a given voltage using a pair of calomel electrodes with salt bridges, which were inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a current amplifier (Keithley, Cleveland OH, model 427) monitored on a storage oscilloscope (Tektronix, Beaverton OR, 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 [27].

Other methods. Mitochondrial protein was measured by the biuret method, using KCN to account for turbidity due to phospholipids [28]. In the purified preparations protein was determined by the Lowry method modified for the presence of Triton [29]. Alternatively, protein was determined by densitometry of SDS-polyacrylamide gels, stained with Coomassie blue, by means of an LKB 2202 Ultroscan laser densitometer coupled to a Shimadzu C-R 1B. In these measurements carbonic anhydrase served as a standard. The estimation of the porin content in whole mitochondria was also obtained by scan densitometry after polyacrylamide gel electrophoresis of SDS-solubilized mitochondria.

Results

Purification of mammalian porins

We have recently identified the 35 kDa DCCD binding protein of pig heart as porin on the basis of its ability to form pores in lipid bilayer membranes [18]. The method employed for the isola-

tion of this protein has the advantage of using whole mitochondria as starting material, instead of isolated outer membranes, as was the case for the only previously purified mammalian porin, i.e., that of rat liver [9]. We have now purified several porins from mammalia by applying the same purification procedure adopted for the isolation of porine from pig heart.

As documented in Fig. 1, all the mammalian porins so far isolated consist of a single protein band when analyzed by SDS gel electrophoresis. Furthermore, Fig. 1 shows that mammalian porins have a very similar electrophoretic mobility. Thus porins from rat brain, rat heart, rat kidney and rat liver, from bovine heart and from rabbit kidney and rabbit liver have a molecular mass of 35.5 kDa. Only porins isolated from pig-heart and pig-kidney mitochondria have a slightly lower M_r (35000). Interesting, the molecular weight of the mitochondrial porins from lower eukaryotic cells varies more widely. Thus the porin from yeast has an M_r of 30000 and that from *Paramecium* an M_r of 37000 (Fig. 1).

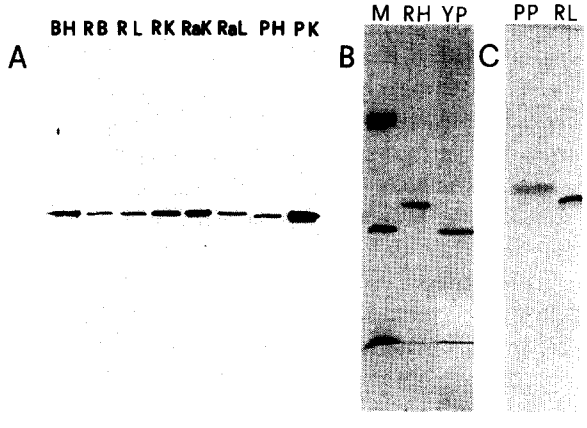

Fig. 1. SDS-polyacrylamide gel electrophoresis of eukaryotic porins. (A) SDS-polyacrylamide gel electrophoresis as described in Materials and Methods; (B) 10% acrylamide SDS-polyacrylamide gel electrophoresis (acrylamide/bisacrylamide = 30:0.8, w/w); (C) 14% acrylamide SDS-polyacrylamide gel electrophoresis (acrylamide/bisacrylamide = 30 : 0.8, w/w). BH: bovine-heart porin; RB: rat-brain porin; RL: rat-liver porin; RK: rat-kidney porin; RaK: rabbit-kidney porin; RaL: rabbit-liver porin; PH: pig-heart porin; PK: pig-kidney porin. M: molecular-weight markers (from the top to the bottom): bovine serum albumin (68000), carbonic anhydrase (30000) and cytochrome *c* (12500); RH: rat-heart porin; YP: yeast porin; PP: *Paramecium* porin; RL: rat-liver porin. 5–10 μg of each porin were applied to the gel. BH, RB, RL, RK, RaK, RaL migrated with an apparent M_r of 35500; PH and PK with an apparent M_r of 35000. Silver staining in A and Coomassie blue staining in B and C.

The amount of pure porins as obtained from 80 mg mitochondrial protein of different tissues was investigated. The use of two different methods, Lowry and scan densitometry (see Materials and Methods), showed that the yield of porin varied between 0.1 and 0.5 mg. With both methods values obtained from kidney were higher than from other tissues. This was found for rat, rabbit and pig. The result may be explained by a somewhat higher porin content of kidney mitochondria. To test this possibility we investigated the relative amount of porin as compared with the total mitochondrial proteins. This was done by measuring the relative amount of the protein bands which corresponded to the pure proteins (Fig. 2). It is easy to see that the amount of porin changed from tissue to tissue: clear bands corresponding to the isolated porins are visible in rat heart, kidney and brain, but almost nothing can be seen in rat-liver mitochondria. By scan densitometry of SDS-solubilized mitochondria after gel electrophoresis we have found that the mitochondrial

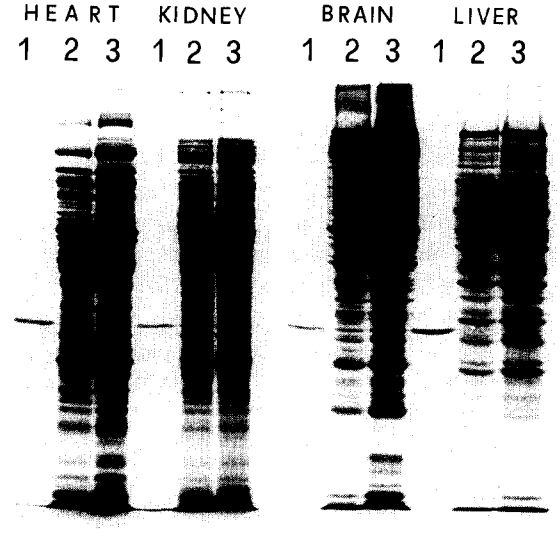

Fig. 2. SDS-polyacrylamide gel electrophoresis of SDS-solubilized mitochondria and of purified porins. 2–3 μg of purified porins (1), 100 μg (2) and 200 μg (3) of SDS-solubilized mitochondria from rat heart, rat kidney, rat brain and rat liver were applied to the gel. Coomassie blue staining.

content of porin is 1.5% in rat kidney, 1% in rat heart, 0.9% in rat brain and 0.5% in rat liver. Our figure of the porin content in rat liver mitochondria closely corresponds to that previously obtained by a different method for the same mitochondria [30].

Peptide mapping

In order to investigate structural differences among the various mammalian porins we have studied the products formed by fragmenting the purified porins with different methods. In Fig. 3 the peptide maps obtained by the *S. aureus* proteinase V 8 and papain are presented. In these experiments the protein bands of SDS gels corresponding to different porins were cut out, equilibrated for 30 min and re-run in the presence of *S. aureus* proteinase V8 or papain. The proteinase V8 is specific for the peptide bonds formed by aspartic or glutamic acid at the COOH-terminal side [31]. Under the experimental conditions of Fig. 3A, six distinct peptides are produced by proteinase V8 degradation of all the mammalian porins tested, these six being the same for every porin. There are also two other polypeptides, due, however, to the presence of proteinase V8 (cf. lane V8 of Fig. 3A). When the degradation of porins by *S. aureus* proteinase V8 reaches completion, e.g., in the presence of greater amounts of proteinase V8 (not shown), only three peptides with an apparent molecular mass of 15, 10 and 7 kDa and corresponding to those indicated by the arrows in Fig. 3A, are obtained from all mammalian porins. Unlike the *S. aureus* proteinase V8, papain is a relatively unspecific proteinase. However, also the peptides produced by papain degradation of all mammalian porins are identical for each porin (Fig. 3B). In these experiments the amount of papain used was carefully chosen in order to obtain a limited number of peptides. Under the conditions of Fig. 3B papain produces, from all mammalian porins, six peptides, two of which are more intensively stained. When a more extensive degradation of porins is reached by using a higher concentration of papain (not shown), only one diffused band is seen near to the front of the solvent. This again holds true for each mammalian porin. Thus the peptide maps of mammalian porins produced by *S. aureus* proteinase V8 and papain indicate a very strong structural similarity among the various mammalian porins.

Fig. 4A and B shows a comparison of peptide maps of porins isolated from mammalia, yeast and *Paramecium*. Whereas the peptide pattern of the rat kidney porin is the same as that produced by proteinase V8 digestion of all mammalian porins, those of porins from yeast and *Paramecium* are

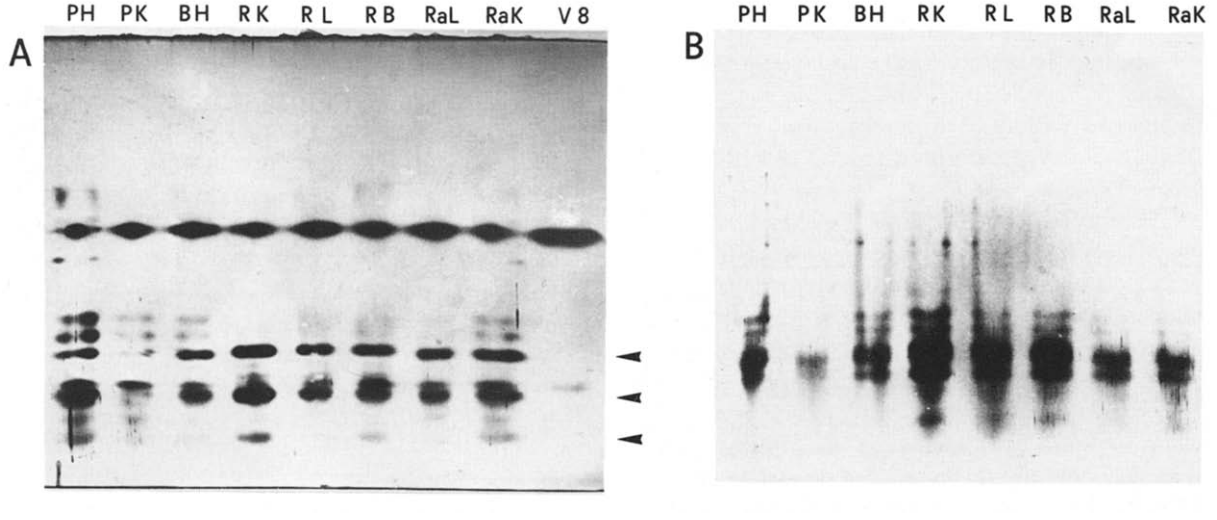

Fig. 3. Peptide maps of mammalian porins obtained by incubating gel slices corresponding to purified porins with *Staphylococcus aureus* proteinase V8 and papain. Gel slices containing 1–3 μg of purified porins were re-run in the presence of 1 μg *S. aureus* proteinase V8 (A) or 0.1 μg papain (B). Silver staining. The abbreviations are as in Fig. 1. The three peptides indicated in A by the arrows are those produced by complete degradation of mammalian porins by proteinase V8.

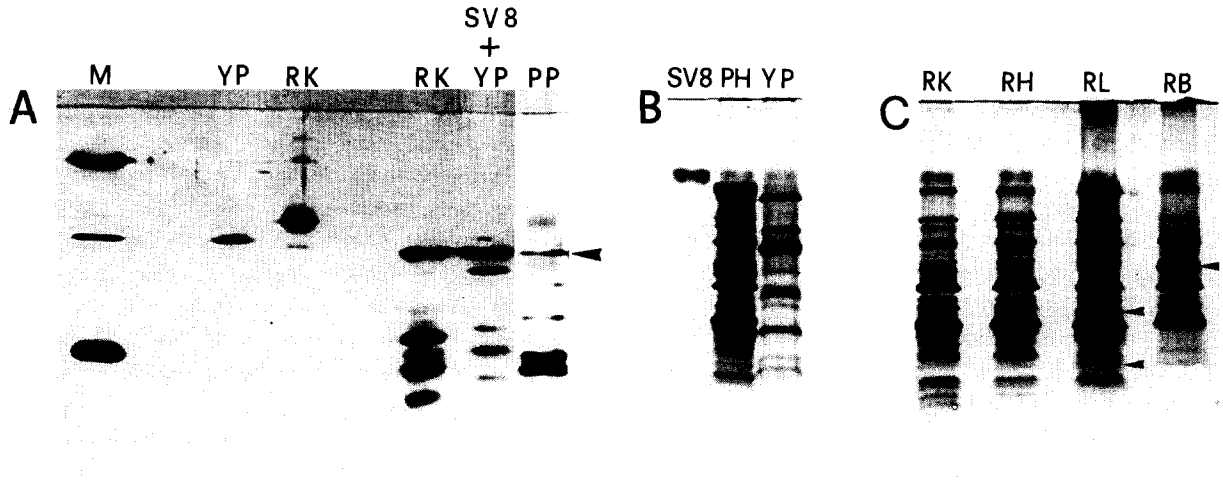

Fig. 4. Comparison of peptide maps of mammalian and yeast porins. (A) Degradation of purified yeast, *Paramecium* and rat-kidney porins in gel slices by *S. aureus* proteinase V8. M: markers (bovine serum albumine – M_r, 68000; carbonic anhydrase – M_r, 30000; cytochrome *c* – M_r, 12500); YP: yeast porin; PP: *Paramecium* porin (same conditions,but different gel as the other lanes); RK: rat-kidney porin; SV 8: *S. aureus* proteinase V8. The arrow indicates the position of the proteinase V8 in the gel after the electrophoretic run. (B) In vitro degradation of yeast and pig-heart porins by *S. aureus* proteinase V8. 10 μg of porins were incubated with 0.5 μg proteinase V8 at 37 °C for 4 h. SV8: *S. aureus* proteinase V8; PH: porin from pig-heart; YP: porin from yeast. (C) In vitro degradation by *S. aureus* proteinase V8 of porins purified from different tissues of rat. Conditions of incubation as in B. Abbreviations as in Fig. 1. The arrows indicate peptides which are different in the various peptide maps. Silver staining.

quite different (Fig. 4A). Thus one high and three low M_r peptides, not corresponding to any of those produced from mammalian porins, are formed from yeast porin when digested by proteinase V8 (lane SV 8 + YP of Fig. 4A). In the case of porin from *Paramecium*, two main peptides of approx. 10 and 12 kDa and two other faint higher M_r peptides are obtained, which point out a marked structural difference from both mammalian and yeast porins (lane SV 8 + PP of Fig. 4A).

The proteolytic digestion carried out on slices of SDS gel [23], as in the experiments of Figs. 3 and 4A, excludes the possibility of fragments derived from contaminant, non stainable polypeptides possibly present in the samples. On the other hand, in this method the mesh net of the gel and the folding of the proteins may limit the action of the proteinases on their substrates. It was therefore interesting to compare peptide maps of mammalia and yeast porins obtained by in vitro proteolysis. The results of these experiments, presented in Fig. 4B, clearly show that the peptide patterns of pig heart and yeast porins are very different also when the proteins are digested in vitro by *S. aureus* proteinase V8. In contrast peptide maps of porins from rat kidney, rat heart, rat liver and rat brain obtained by in vitro proteinase V8 digestion are strikingly similar (Fig. 4C). Out of the many fragments produced by the in vitro proteolysis, there are only two in the case of the liver porin (see arrows) which are different from those produced by rat kidney, rat heart and rat brain. Furthermore, there is only one fragment (see arrow) which is quantitatively more relevant in the brain porin than in the porin from the other tissues. The in vitro proteolysis by *S. aureus* proteinase V8 also reveals that there are some differences between the heart porins from pig and rat, and that these differences are more marked than those existing among the various porins from rat (cf. Fig. 4B lane PH and Fig. 4C).

Immunoblotting

The antigenic properties of the mammalian porins studied in the present investigation were tested by immunoblot. After SDS gel electrophoresis the purified porins were transferred to

nitrocellulose and immunodecorated with a monospecific antiserum raised against rat-liver porin purified from isolated mitochondrial outer membranes. Fig. 5 shows that the antibody against the rat-liver porin reacts with all mammalian porins so far isolated, independently of the tissue and the species, indicating that they all have antigenic properties in common. In contrast, there is no cross-reactivity with porin from yeast (not shown). Interestingly, as documented in Fig. 5 (lanes PH′), the antibody against rat-liver porin reacts also with mammalian porins to which DCCD has been bound as described in previous papers [18,32,33]. This indicates that the binding of DCCD to porin, which has been shown to inhibit the reaction of this protein with hexokinase [33], does not modify the structure of porin so extensively as to cause a loss of its immunological specificity.

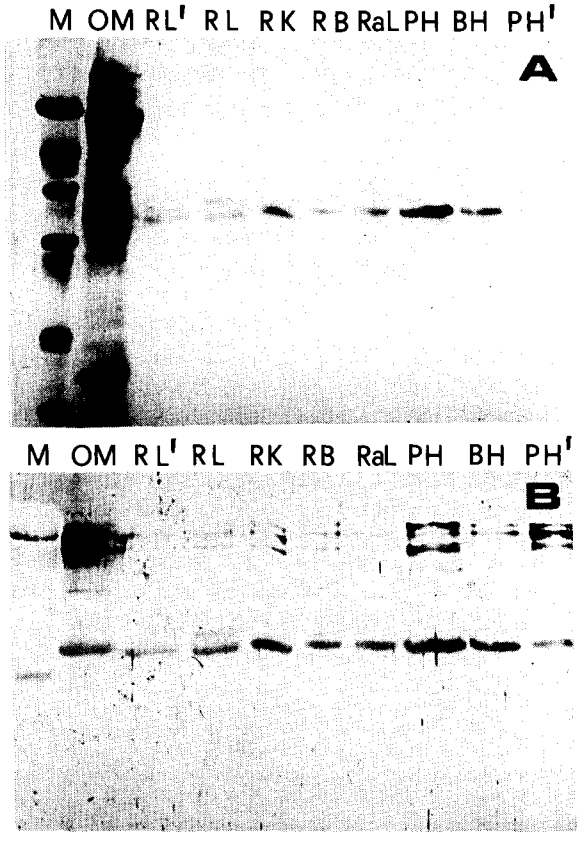

Fig. 5. Immunological cross-reactivity of mammalian porins studied by using the antibody raised against the rat liver porin purified from mitochondrial outer membranes. (A) Protein stain with Ponceau S after electrotransfer of the samples from the gel to a nitrocellulose filter. (B) Immunodecoration of the nitrocellulose filter. M: Pharmacia low-molecular-weight markers. OM: rat-liver outer mitochondrial membranes. RL′: rat-liver porin purified from isolated mitochondrial outer membranes. PH′: 35-kDa-DCCD labelled porin from pig heart mitochondria. Other abbreviations as in Fig. 1.

Macroscopic conductance

When one of the mitochondrial porin preparations was added in small quantities (10–100 ng/ml) to the aqueous solutions bathing a lipid bilayer membrane, the conductance of the membrane increased by several orders of magnitude. The time-course of the conductance increase was similar to that described previously for bacterial porins [34]. After an initial rapid increase for 15–20 min, the membrane conductance increased at a much slower rate, and this slow increase continued until membrane breakage. The conductance increase occurred regardless of whether the protein was added to only one side or to both sides of the membrane. The addition of the detergent alone at the same concentrations as present in the protein solutions did not lead to any appreciable increase in the membrane conductance above the specific conductance in the absence of protein (10^{-8}–10^{-7} S/cm^2).

Since a steady conductance level could not be reached in these experiments, the dependence of the membrane conductance on the protein concentration was difficult to determine. However, by using the conductance values measured at a fixed time (30 min after the addition of the protein) it was possible to show a linear relationship between the protein concentration in the aqueous phase and the specific membrane conductance. Under these conditions a comparison of the macroscopic conductance of different porins was made. The specific conductance as obtained from a protein concentration of 50 ng/ml varied about 5-fold between $1 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ S/cm^2 for the different porins.

Single-channel analysis

The addition of smaller amounts of the mitochondrial porin preparations (1–10 ng/ml) to the lipid bilayer membranes having small surface areas (0.1 mm^2) allowed the resolution of step increases in conductance. Most current steps were

directed upward and terminating steps were only rarely observed at a transmembrane potential of 10 mV indicating a long lifetime of the pores at small voltages. Table I compares the average single channel conductance $\overline{\Lambda}$ of the different mitochondrial porins. With the exception of the porin from *Paramecium* mitochondria, the values are very similar. The absence of any binding site and the high single-channel conductance allow a rough estimate of the effective diameter of the mitochondrial pores. Assuming that the pores are filled with a solution of the same specific conductivity, σ, as the external solution and assuming a cylindrical pore with a length l of 6 nm, the effective pore diameter d ($= 2r$) can be calculated according to

$$\overline{\Lambda} = \frac{\sigma \pi r^2}{l} \quad (1)$$

As shown in table I, the pore diameter of all mammalian porins tested is 1.7 nm. The porin from *Paramecium* shows a diameter of the channel much lower (1.3 nm) and that from yeast the same as that of the mammalian porins.

TABLE I

AVERAGE SINGLE-CHANNEL CONDUCTANCE $\overline{\Lambda}$ OF DIFFERENT MITOCHONDRIAL PORINS

The solutions contained 1–10 ng/ml mitochondrial porin, 1 M KCl and less than 0.1 μg/ml Triton X-100; the pH was between 6.0 and 7.0. The membranes were made from a mixture diphytanoyl phosphatidylcholine and phosphatidylserine in *n*-decane (1%, w/v), $T = 25\,^{\circ}$C; $V_m = 10$ mV. $\overline{\Lambda}$ was determined by recording at least 70 conductance steps. The pore diameter was calculated according to Eqn. 1.

Source of mitochondrial porin	$\overline{\Lambda}$ (nS)	Pore diameter (nm)
Rat heart	4.0	1.7
Rat liver [a]	4.3	1.7
Rat kidney	4.0	1.7
Rat brain [b]	4.0	1.7
Rabbit liver	4.0	1.7
Rabbit kidney	4.0	1.7
Bovine heart	4.0	1.7
Pig heart	3.5	1.6
Pig kidney	4.0	1.7
Paramecium	2.4	1.3
Yeast	4.2	1.7

[a] Value is in agreement with that reported in Ref. 10.
[b] Value is in agreement with that reported in Ref. 38.

Selectivity of mitochondrial porins

Further information about the structure of the pore formed by the mitochondrial porins isolated form different mammalian tissues and from yeast and *Paramecium* was obtained by zero-current membrane potential measurements in the presence of salt gradients. Table II shows the results of the measurements for a 10-fold gradient of KCl. The potentials were found to be negative on the dilute side, indicating preferential movement of Cl^-. The zero-current membrane potentials were analyzed using the Goldman-Hodgkin-Katz equation [27]. The ratio of the anion permeability P_a divided by the cation permeability P_c suggests that Cl^- has a higher mobility inside the pore than K^+ despite the same mobility in the aqueous phase [35]. The small anion selectivity may be explained by an excess of positively charged groups in or near the pore [11,36].

Voltage dependence

The mitochondrial porins studied so far are voltage-dependent and switch to substates of

TABLE II

ZERO-CURRENT MEMBRANE POTENTIALS V_m IN THE PRESENCE OF A 10-FOLD CONCENTRATION GRADIENT OF KCl

V_m is the electrical potential on the dilute side minus the potential on the concentrated side. The membranes were formed from diphytanoylphosphatidylcholine/*n*-decane. The aqueous solutions were unbuffered and had a pH of about 6. The ratio of the permeabilities P_c (cation) and P_a (anion) was calculated according to the Goldman-Hodgkin-Katz equation [27].

Source of mitochondrial porin	V_m (mV)	$\frac{P_c}{P_a}$
Rat heart	0	1
Rat liver [a]	−2	0.91
Rat kidney	−11.3	0.58
Rat brain [b]	−11	0.60
Rabbit liver	−6	0.75
Rabbit kidney	0	1
Bovine heart	−6.7	0.73
Pig heart [c]	−10	0.62
Pig kidney	0	1
Paramecium	−11.4	0.56
Yeast [d]	−7	0.71

[a] Value is in agreement with that reported in Ref. 10.
[b] Value is in agreement with that reported in Ref. 38.
[c] Value is in agreement with that reported in Ref. 18.
[d] Value is in agreement with that reported in Ref. 19.

reduced conductance at higher transmembrane potentials [11,20,37]. For the porins isolated from rat liver and pig heart less than 50 mV are sufficient to reduce the initial conductance to about 60%. In the case of rat brain, on the other hand, a transmembrane potential of more than 100 mV is necessary for the same effect [38]. In this study, we have investigated the voltage dependence of other mammalian and low eukaryotic porins in detail. All were found to be voltage-dependent and a bell-shaped curve was obtained for the ratio of the conductance, G, at a given membrane potential devided by the conductance, G_0, at zero potential as a function of the applied membrane potential, V_m. These curves were analysed using the equation:

$$N_o/N_c = e^{nF(V_m - V_o)/RT} \quad (2)$$

where F, R and T have the usual meaning, N_o/N_c is the ratio of open-to-closed channels, n is the number of gating charges moving through the entire membrane potential gradient, V_m, for channel gating and V_0 is the potential where 50% of the total number of channels are in the closed configuration.

TABLE III

DATA OF THE NUMBER n OF GATING CHARGES AND V_0, THE VOLTAGE, WHERE 50% OF THE CHANNELS ARE IN THE CLOSED CONFIGURATION FOR PORINS OF DIFFERENT TISSUES

The measurements were carried out in a solution of 1 M KCl and 50 ng/ml mitochondrial porin with membranes of 1% diphytanoylphosphatidylcholine/phosphatidyl-serine 4 : 1 in n-decane.

Source of mitochondrial porin	n	V_0
Rat liver	2.3	55
Rat kidney	2.5	40
Rat brain [a]	1	60
Rabbit liver	1.8	67
Rabbit kidney	2.5	38
Bovine heart	1.6	62.5
Pig heart	2	40
Pig kidney	3.4	35.5
Paramecium	2	32
Yeast	2	24

[a] Value is in agreement with that reported in Ref. 38.

Table III shows the data for n and V_0 derived from the ratio G/G_0 as published earlier [38,5]. The number of gating charges was approx. 2–4 for most eukaryotic porins, including those from yeast and *Paramecium*. However, for porins from rat brain and bovine heart n was significantly lower, which indicated that these porin pores were less voltage dependent. Subsequently, for these pores, the potential, V_0 was shifted to higher positive membrane potentials.

Discussion

We have isolated and purified the mitochondrial porins of a variety of mammalian tissues and of some low eukariotic cells. A relatively simple method starting from whole mitochondria was used for the purification procedure which allowed the isolation of pure porins with a high yield. The non-adsorptivity to resins like hydroxyapatite may be caused by the fact that the porins, similar to the carriers of the mitochondrial inner membrane [39], are deeply buried in the outer membranes and are very hydrophobic. This idea is consistent with the experimental result that proteases hydrolyse most proteins of the mitochondrial outer membrane but not the porin [14]. The yield of the purified protein was especially high starting from kidney mitochondria, which seem to contain a higher amount of porin (Fig. 2). When applied to SDS gel electrophoresis, all the mammalian porins isolated in this study consist of a single protein band. Those purified from pig-heart and pig-kidney showed an M_r of 35 000 while all the others a slightly higher M_r (35 500). The porins from lower eukariotic mitochondria showed, on the other hand, a more pronounced variability, the molecular weights being 30 000 in yeast and 37 000 in *Paramecium* (Fig. 1).

The peptide mapping showed that the mitochondrial porins of the different mammalian tissues are closely related to one another, since the peptide patterns were very similar (Figs. 3 and 4). On the other hand, we found some differences between the patterns of some porins (Fig. 4C). This result suggests that the porins of different tissues of the same organism showed some differences in their primary sequence. In fact, the electrophysiological studies of rat-brain porin and

rat-liver porin also showed that both porins contain a small but distinct difference either in the protein sequence or in the pore-forming complex (Tables II and III). Furthermore, rat-liver and rat-brain mitochondria bind hexokinase with different affinity, which could be due to differences in the binding sites of the porins or to differences in the hexokinases present in the two tissues [40]. It has to be noted, however, that these differences are small and may only consist in changes of few amino acids because antibodies against rat-liver porin cross-reacted with all the other mammalian porins but not with yeast porin (Fig. 5). This result indicated that the structural differences between yeast porin and mammalian porins were more substantial, which was also reflected in the completely different peptide maps (see Fig. 4A and B). The peptide pattern of the porin from *Paramecium*, the other lower eukaryotic tested in this study, is also completely different from that of the mammalian porins (Fig. 4A). This result, together with the finding that there is no obvious homology between the sequences of yeast and *Escherichia coli* porins [15], suggests that major changes in the primary structure of the pore proteins may be expected climbing the evolutionary stairs from bacteria to mammalia.

Despite all the differences in protein chemistry described above, we found a clear analogy of the pore properties between all mitochondrial porins studied so far in electrophysiological experiments. The single-channel conductance was for all porin pores, except for that of *Paramecium*, about 4 nS in 1 M KCl (Table I). Assuming that the mitochondrial pore is in the open state a so-called general diffusion pore, similar to most bacterial porins [20], the pore diameter may be estimated to be around 1.7 nm. This value is in good agreement with the results of research into the permeability of isolated mitochondria [2] and with those of research into the electron microscopy of mitochondrial outer membranes [41,42]. A similar close analogy was also found for the voltage dependence of the mitochondrial pores (Table III). The mitochondrial porins from mammalian tissues, yeast and *Paramecium* formed voltage-dependent pores which switched to substates at transmembrane potentials higher than 10 mV, similarly to the mitochondrial porin of *Neurospora crassa* [12]. This strong analogy of the pore properties forces us to believe that the function of the pore was conserved during the evolution of the eukaryotic cells despite all possible variations of the primary structure of the pore-forming proteins. It is interesting that the properties of the bacterial porins are very different from the mitochondrial ones. Thus, the former show pores which are smaller, are usually cation selective and are not influenced by the applied voltage (see Ref. 11 for a review). The *Paramecium* mitochondrial porin and the *Paracoccus denitrificans* porin [43] show intermediate properties between the eukaryotic and the prokariotic porins. The *Paramecium* pores have a diameter similar as in bacteria. The *Paracoccus* pores, on the other hand, are nearly as large as the mitochondrial ones. The protein from *Paracoccus* forms dimers, as it has been proposed for a mitochondrial porin [44], and not trimers as has been found for bacterial porins [11].

The size of the pore of the mitochondrial porins is apparently reduced at higher voltages. This would result in the restriction of the permeability of the pore for larger solutes. Furthermore, we could recently show (Ludwig, O. and Benz, R., unpublished results) that the mitochondrial porin of *Paramecium* is highly cation selective in the closed state. This would mean that the substrates of mitochondria (which are mostly anionic) are excluded from the pore and cannot enter these organelles if the pores are in the closed state. Thus, a transmembrane potential across the mitochondrial outer membrane could control the metabolism of mitochondria [38].

It is now well established that kinases such as hexokinase and glycerokinase bind to the porin, i.e., the hexokinase-binding protein [45–47]. Both enzymes utilize directly the ATP coming from the mitochondrial ATP pool. In fact, it has been shown in the literature [48] that hexokinase bound to the surface of mitochondria has a higher activity with ATP coming from oxidative phosphorylation as compared with ATP added to the cytoplasmic side. This observation led to the suggestion that the pore plays also an important role in the formation of the mitochondrial microcompartment described for hexokinase and kreatine kinase [49,50]. The microcompartment formed between hexokinase and adenylate translocator in energized mito-

chondria excludes adenylate kinase (located in the intermembrane space) from the mitochondrial ATP-pool [3].

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