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Characterization of pore-forming activity in liver mitochondria from *Anguilla anguilla*. Two porins in mitochondria?

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A fast purification procedure for the isolation and purification of eukaryotic porin (De Pinto et al., (1987) Biochim. Biophys. Acta 905, 499–502) was applied to liver mitochondria of the fish *Anguilla anguilla*. A protein preparation was obtained which formed slightly anionically selective pores in reconstitution experiments with lipid bilayer membranes. The distribution of single-channel conductances had two maxima of 2.4 nS and 4.0 nS in 1 M KCl. Sodium dodecylsulfate electrophoretograms of the protein preparation showed the presence of two bands of very similar electrophoretic mobility (32 and 32.5 kDa). Both bands cross-reacted with antibodies raised against purified bovine heart porin and with antibodies raised against the 19 amino acids N-terminal end of human porin. No cross-reactivity was observed with antibodies against yeast porin. The peptide maps of the two bands showed slight differences. The possibility of the presence of two different porins in liver mitochondria of *Anguilla anguilla* is discussed. An extensive immunological comparison of different mitochondrial porins is presented.

Introduction

The mitochondrial outer membrane is known to be freely permeable to hydrophilic solutes up to a well-defined molecular size caused by the presence of a general diffusion pore [1]. The protein responsible for the permeability properties of the mitochondrial outer membrane was named porin [2] or VDAC (voltage-dependent anion-selective channel) [3]. The channel-forming protein was isolated from mitochondrial outer membrane and whole mitochondria [4,5]. Reconstituted in artificial lipid membranes it forms large slightly anionically selective pores in the open state. The pores are voltage-dependent and switch to substates of completely different permeability and selectivity [6]. Furthermore, porin was identified as the hexokinase-binding protein, suggesting that it may play an important role in the regulation of mitochondrial metabolism [7,8]. The pore-forming properties, the biosynthesis and the primary structure of the mitochondrial porins from *Neurospora crassa* and from *Saccharomyces cerevisiae* are known in detail. Neither porin has a particularly hydrophobic primary structure and, according to predictions derived from the hydrophobic profiles, the secondary structure is mainly composed of membrane-spanning sided β-sheets [9,10]. This finding has recently been confirmed in the human porin [11]. We have recently characterized and compared several porin pores from advanced and primitive eukaryotic cells [5,6,12,13]. Immunological and peptide-mapping experiments show strong structural similarities among mammalian porins. The pore-forming proteins isolated from low eukaryotic organisms, on the other hand, show different peptide maps and no immunological cross-reactivity with the mammalian porins [5,6].

The outer membrane of yeast mitochondria contains, besides the 'normal' porin-pore with an apparent diameter of 1.7 nm, a smaller cationically selective general diffusion pore. This pore has recently been observed, when the gene encoding for porin is deleted [14,15].

Abbreviations: SDS, sodium dodecylsulfate; HTP, hydroxyapatite; VDAC, voltage-dependent anion channel.

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This means that, as in Gram-negative bacteria, yeast has at least two genes coding for proteins with pore-forming activity in the outer mitochondrial membrane.

In this paper, we applied the same purification procedure used for mammalian porins [16,17] to mitochondria obtained from the liver of the fish *A. anguilla*. The procedure resulted in a protein preparation which was able to form ion-permeable pores, when reconstituted into artificial lipid membranes, with estimated diameters of 1.2 and 1.7 nm. Immunological cross-reactivity and peptide maps indicated that the protein preparation is the porin, although the electrophoretograms showed two bands of very similar molecular masses. This is the first report of porin(s) from fish.

Materials and methods

Materials

Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad, Triton X-100, acrylamide and *N,N'*-methylenebisacrylamide from Serva. Celite 535 was purchased from Roth, *Staphylococcus aureus* V8 proteinase from Miles laboratories and papain from Sigma.

Isolation of Anguilla anguilla liver mitochondria and purification of porin

Yellow eels (*A. anguilla*), grown in seawater, were purchased from Ittica-Ugento (Italy) and kept in seawater aquaria. The fish were decapitated: the livers were removed immediately and put in 0.25 M sucrose, 10 mM Tris-HCl, 3 mM EDTA, 5 mM 2-mercaptoethanol (pH 7). The livers were cut into small pieces, washed several times in the same buffer and homogenized in a Teflon-glass homogenizer (with 2 ml of buffer per g of liver). The homogenate was then filtered through gauze and centrifuged at $800 \times g$ for 8 min. The supernatant was centrifuged for 10 min at $15000 \times g$; the resulting pellet was rehomogenized in sucrose buffer and centrifuged for 10 min at $8000 \times g$. The isolated mitochondria were frozen or immediately used for the protein purification.

The purification of *A. anguilla* porin was performed essentially as in ref. 16. The mitochondria were lysed by osmotic shock; after centrifugation the pellet, composed mainly of mitochondrial membranes, was solubilized by 3% Triton X-100, 10 mM Tris-HCl (pH 7.0) and 1 mM EDTA. The final concentration was 15 mg protein/ml. In some control experiments the following cocktail of proteinase inhibitors was present: 10 μM leupeptin, 250 μM phenylmethanesulfonyl fluoride (PMSF), 10 μM pepstatin. After 30 min at 0°C, the solubilization mixture was centrifuged at $40000 \times g$ for 15 min and the supernatant (0.6 ml) was loaded onto a dry hydroxyapatite/celite column (0.6 g, ratio 2:1). The elution was performed with the solubilization buffer. The first 0.6 ml eluted were collected.

SDS-gel electrophoresis and peptide mapping experiments

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS. The separation gel contained routinely 14% acrylamide with a ratio acrylamide/bisacrylamide of 30:0.8. 17.5% acrylamide with a ratio acrylamide/bisacrylamide of 30:0.2 was used to achieve a better resolution in the mass region of 30–35 kDa. Other details as in Ref. 18. The proteolysis was performed on protein bands of Coomassie blue-stained SDS gel as in Ref. 19.

Immunoblotting experiments

Antiserum against bovine heart porin was obtained from rabbit. After a first injection of 200 μg of purified porin mixed with Freund's complete adjuvant, two more portions of 200 μg of porin were injected after 3 and then 2 weeks. Blood was taken from the rabbit ear.

The proteins separated on a 14% SDS-PAGE were transferred to nitrocellulose [20,21], incubated with the antisera and then with an anti-rabbit Ig horseradish peroxidase linked antibody (purchased from Amersham). The peroxidase reaction was performed by 20 ml of a mixture of 0.05% 4-chloro-1-naphthol, 16% methanol, 0.5% BSA in 0.14 M NaCl, 0.01 M phosphate (pH 7.0) with the final addition of 12 μl of 30% H_2O_2. The antiserum against the N-terminal part of human porin was a kind gift of F. Thinnes (Göttingen). The antiserum against yeast porin was a kind gift of M. Dihanic (Basel), and that against *N. crassa* of R. Kleene (Munich).

Lipid bilayer experiments

The methods used for the black lipid bilayer experiments have been described previously [22]. The membranes were formed across a circular hole (surface area about 0.1 mm^2) in the thin wall separating two aqueous compartments from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. Bilayer formation was indicated when the membrane turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. The macroscopic conductance measurements were performed on larger membranes (1 mm^2) with a Keithley 610 C electrometer. Zero-current membrane potentials were measured with the same instrument 5–10 min after the application of a 5-fold salt gradient across the membranes [23].

Other methods

Mitochondrial protein was measured by the biuret method, using KCN to account for turbidity due to

phospholipids [24]. Purified protein was measured by the Lowry method modified for the presence of detergent [25].

Results

Purification and immunological characterization of A. anguilla porin(s)

We have recently published a rapid method for the purification of mammalian porins [16]. The advantage of the method lies in its single chromatographic step on a hydroxyapatite/celite column after the solubilization of the mitochondria by the non-ionic detergent Triton X-100. The choice of appropriate conditions during the solubilization and the chromatography allowed the elution of a single protein, namely the porin, from a large number of different mitochondrial membrane proteins [16]. Fig. 1A shows the results obtained by applying the same purification method to mitochondrial membranes from bovine heart and from yellow eel (*A. anguilla*) liver. A single band is visible in the right lane corresponding to the protein eluted from the hydroxyapatite/celite column in the case of bovine heart mitochondria. The central lane shows the eluate obtained from the same kind of chromatography for eel mitochondria in which two very close bands were observed. The apparent molecular masses of these two bands were 32500 and 32000, respectively, as determined by comparison to Bio-Rad low-molecular-weight markers. Elution of these two protein bands was reproducibly observed from several mitochondrial preparations. The quantitative ratio of the two proteins, determined by scan densitometry, was usually 1:4, upper to lower protein band (Fig. 1A). The same result was also obtained in control experiments in which a mixture of different proteinase inhibitors was added during the isolation and purification procedure. To exclude the possibility of the contamination of the porin fraction by other proteins, it was analyzed immunologically. Both proteins clearly reacted with the antiserum raised against the bovine heart porin (Fig. 1B).

A comparative immunological analysis of *A. anguilla* porin(s) is shown in Fig. 2. The electrophoresis was performed in 14% acrylamide gels, in which the separation of the two bands was not so clear, although still visible when the electrophoretograms were stained by Coomassie blue (panel A). The intensity of the immunostain usually covered the appearance of two bands in the immunoblotting experiments (panels B to E). Western blots of purified porins from bovine heart, yeast and eel were immunodetected with antibodies raised against the *S. cerevisiae* porin (panel B), against the synthetic 19-amino-acids long N-terminal part of human porin (panel C) [26], against the bovine heart porin (panel D), and against the *N. crassa* porin (panel E). The porin from eel liver cross-reacted with antibodies raised

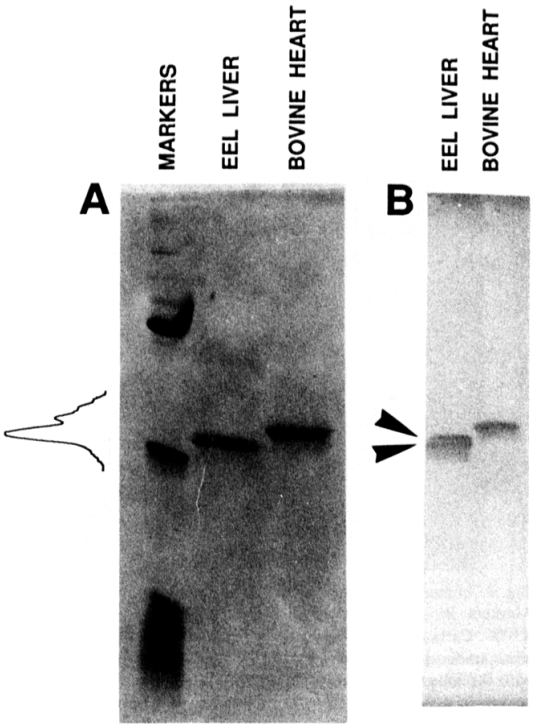

Fig. 1. (A) SDS-PAGE of hydroxyapatite/celite eluates of solubilized mitochondria from bovine heart and *A. anguilla* (eel). The acrylamide concentration was 17.5% (ratio acrylamide:bisacrylamide = 30:0.2). The *A. anguilla* porin was split into two bands. On the left side of the panel the densitometric scan of the two bands is shown. Markers: molecular weight markers (from top to bottom: bovine serum albumin, 68000; carbonic anhydrase, 30000; cytochrome *c*, 12500). The right panel (B) shows the Western blot of the same gel. The two eel bands were both immunostained (see arrows).

against bovine heart porin but not with antibodies raised against porins from lower eukariotic organisms, namely from *S. cerevisiae* and *N. crassa*. Interestingly, eel porin cross-reacted also with antibodies raised against the N-terminal part of human porin. The results of panels B to E show also that antibodies against yeast porin reacted only with the porin purified from the same organism and that antibodies against *N. crassa* were only active against yeast porin, and then needed at a very high titer.

The availability of antisera against different porins allowed us to perform an extensive comparison of the immunological similarities among eukaryotic porins. This immunological analysis is shown in Table I. A growing sensitivity to mammalian antisera (an antiserum to the purified bovine heart porin and an antiserum to the 19 N-terminal amino acids of human porin) was found as the evolutionary ladder was ascended. Interestingly, both fish and insect porins were

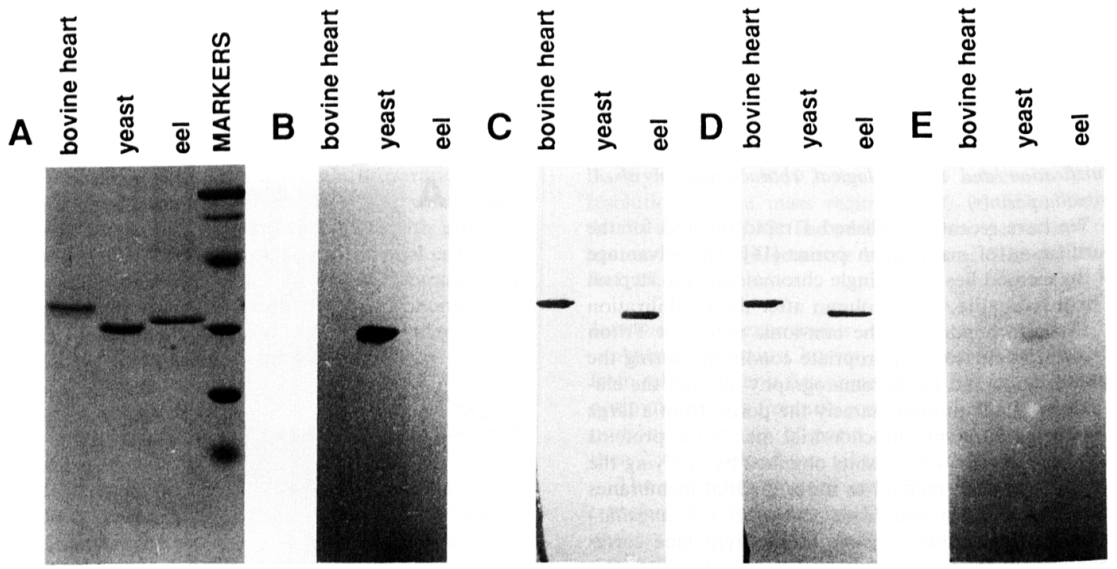

Fig. 2. Immunological analysis of *A. anguilla* porin. (A) The electrophoresis was performed in a 14% acrylamide gel. Coomassie blue staining. Markers: Bio-Rad low-molecular-weight markers (from top to the bottom: Phosphorylase *b*, 92500; bovine serum albumin, 68000; ovalbumin, 45000; Carbonic anhydrase, 30000; soybean trypsin inhibitor, 21500; Lysozyme, 14400). Four gels were run in the same conditions, loading the same amounts of porin obtained from bovine heart, yeast and eel mitochondria. The gels were transferred to nitrocellulose and immunodecorated with the following antisera: (B) 1:800 antibodies against yeast porin; (C) 1:1000 antibodies against the N-terminus of human porin; (D) 1:1000 antibodies against bovine heart porin; (E) 1:100 antibodies against *N. crassa* porin.

detected by the antiserum to the human porin N-terminal end, indicating structural similarities at least in this region. On the other hand, sensitivity to antisera raised against porins purified from primitive organisms (such as *S. cerevisiae* and *N. crassa*) was confined to the lowest steps of the evolutionary ladder (protozoa, fungi and insect).

Peptide maps

Peptide mapping experiments provided more insight into the structure of the two proteins obtained by chromatography of Triton X-100 solubilized mitochondrial membranes from *A. anguilla* liver on a hydroxyapatite/celite column. This was done in the following way: the gel containing the two bands was cut into slices to separate the two proteins and the slices were subjected to proteinase V8 from *S. aureus* (Fig. 3A) and papain (Fig. 3B). The peptide maps of bovine heart porin, obtained with the same proteinases, are shown for comparison. The results indicate that the maps

TABLE I

Immunological comparison of eukaryotic porins

Organism	Channel diameter [a]	Subunit mass [b]	Cross-reactivity with antisera against				Ref.
			BHP[c]	N-term	yeast	*N. crassa*	
S. cerevisiae	1.7	31000	−	−	+ + +	+	13, this work
S. cerevisiae (2nd porin)	1.2	20000?					15
Paramecium tetraaurea	1.3	37000	−	−	+	+ +	27, this work
Drosophila melanogaster	1.7	31000	−	+	+ +	−	6, this work
Anguilla anguilla	1.7, 1.2	32500	+ +	+ +	−	−	this work
Rattus norvegicus	1.7	35550	+ + +	+ + +	−	−	5, this work

[a] Determined in 1 M KCl according to Ref. 22.
[b] Determined by SDS-PAGE.
[c] Abbreviations: BHP, bovine heart porin; N-term, antiserum to the 19 N-terminal amino acids of human porin; yeast, *S. cerevisiae*. The sign − indicates absence of cross-reactivity. The sign + indicates presence of cross-reactivity and the number of + indicates the intensity of the immunological reaction.

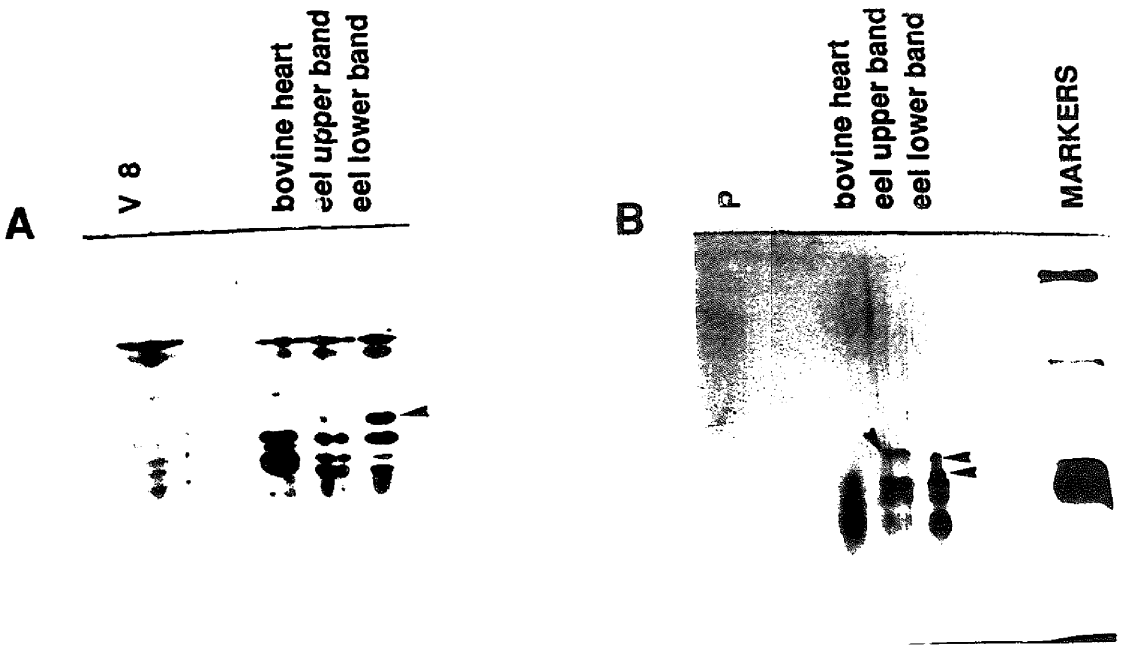

Fig. 3. Peptide maps of the two *A. anguilla* porin bands and of bovine heart porin. Gel slices containing similar amounts (2–3 μg) of purified porin were re-run in the presence of 1 μg *Staphylococcus aureus* proteinase V8 (panel A) or 0.2 μg papain (panel B). Silver staining. Markers: molecular weight markers (from top to bottom: bovine serum albumin, 68000; carbonic anhydrase, 30000; cytochrome *c*, 12500); V8: 1 μg of *S. aureus* proteinase V8; P: 0.2 μg of papain; bovine heart: bovine heart porin; eel upper band: upper band of *A. anguilla* porin preparation; eel lower band: lower band of *A. anguilla* porin preparation. The arrows indicate peptides which are different in the peptide maps of upper and lower protein bands.

obtained from the upper and the lower eel protein bands were similar. In the case of the V8 proteinase the eel proteins were cleaved into four main peptides with virtually the same electrophoretic mobility (Fig. 3A). The main difference was a strong band of a high molecular mass peptide exhibited by the eel lower band which was scarcely visible in the lysate of the upper band. In the case of the rather unspecific proteinase papain (Fig. 3B) the upper and lower bands were differently cleaved. The upper band contained a high molecular mass fragment which was not observed among the fragments of the lower band. Alternatively, the fragments of the lower-molecular mass band contained two peptides which were not present in the fragments of the other band. It is interesting to note that the peptide maps of the eel proteins showed patterns completely different to those of bovine heart porin, which were very similar to those which have been published previously for other mammalian porins [5].

Lipid bilayer experiments

Lipid bilayer experiments were performed to study whether the porin isolated from mitochondria of the fish had any pore-forming activity. In a first set of experimental conditions we demonstrated that protein concentrations of 10 to 100 ng/ml were able to increase the specific conductance of lipid bilayer membranes by many orders of magnitude. The time-course of the increase was similar to that described previously for different mitochondrial and bacterial porins [27]. The addition of much smaller concentrations of the porins to membranes with a surface of about 0.1 mm^2 allowed the resolution of step increases in membrane conductance (see Fig. 4). These steps were specific for the presence of the fish porin and were not observed when only the detergent Triton X-100 was present at the same concentration in the aqueous phase. They defined the protein preparation (composed of the 32 and 32.5 kDa proteins) as pore-forming components in lipid bilayer membranes. It has to be noted, however, that the single-channel conductance of a certain fraction of pores was smaller (2.4 nS in 1 M KCl) than that of most mitochondrial porins studied to date (about 4 nS in 1 M KCl [5]) with the only exception of *Paramecium* porin [27]. On the other hand, the smaller conductance steps have been found in all reconstitution experiments performed in our laboratory with eukaryotic porins [28]. Fig. 5 shows a histogram of the conductance steps

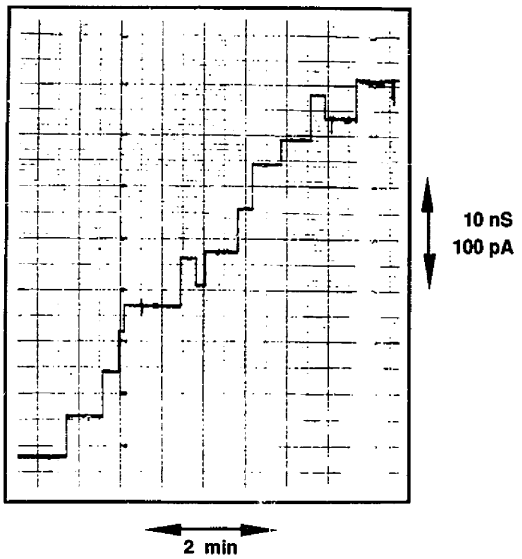

Fig. 4. Single channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membrane after the addition of 5 ng/ml eel porin to the aqueous phase. The aqueous phase contained 1 M KCl (pH 6). The applied voltage was 10 mV; $T = 25°C$.

observed with fish porin in 1 M KCl solution. The maxima of the distribution were located between 2.0 and 3.0 nS and 3.5 and 4.5 nS. We do not think that the small conductance steps are simply half of the large channel, although this possibility cannot completely be excluded. It seems that the smaller channel could represent a different configuration of the same protein as was observed with *Paramecium* porin [27].

The single-channel conductance of the eel porin was studied as a function of the KCl concentration in the aqueous phase. In all experiments two conductance

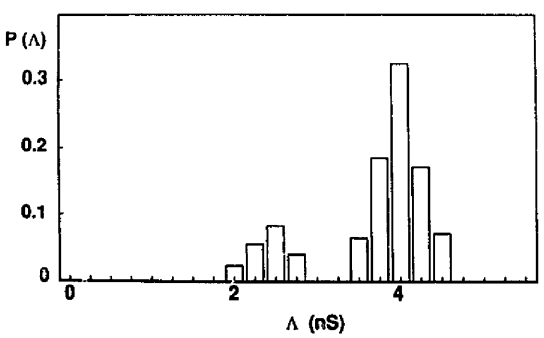

Fig. 5. Histogram of 236 conductance steps observed with diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of eel porin. The mean of the left-hand maximum was 2.4 nS for 58 single events and that of the right-hand maximum was 4.0 nS for 178 single events.

TABLE II

Average single-channel conductance of eel porin in different salt solutions of concentration c

The aqueous solutions contained 5–20 ng/ml porin 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 1% (w/v) solution of diphytanoyl phosphatidylcholine in *n*-decane; $T = 25°C$; $V_m = 10$ mV. Λ was determined by recording at least 90 conductance steps and averaging over the two peaks shown in Fig. 5.

Salt	c (M)	Λ (nS)	
		low-conductance pore	high-conductance pore
KCl	0.01	0.03	0.05
	0.03	0.09	0.15
	0.1	0.28	0.47
	0.3	0.83	1.5
	1	2.4	4.0
	3	6.2	9.9
LiCl	1	1.6	3.0
KCH_3COO	1	1.2	2.4

peaks were found similar to those shown in Fig. 5. The results are summarized in Table II. Both peaks exhibited a linear relationship between single-channel conductance and salt concentration. Single-channel experiments were also performed with LiCl and KCH_3COO instead KCl to gain some insight in the selectivity of the two types of channel formed by the porin of the eel. Again, two peaks were observed in the histograms. The single-channel conductance in 1 M LiCl was always smaller than in 1 M KCH_3COO. This result indicated some preferential movement of anions over cations through both channels because K^+ has the same aqueous mobility as Cl^-, whereas Li^+ has the same mobility as acetate. On the other hand, it is clear that the fish porin forms wide water-filled channels because of the large single-channel conductances and a linear conductance-concentration relationship observed for both peaks in the histograms. The diameter of the so-called general diffusion pores can be estimated by assuming that they are cylinders with length l and diameter d and that they are filled with a solution which has the same specific conductivity, σ, as the bulk aqueous solution. In this case, the single-channel conductance, Λ, is given by:

$$\Lambda = \sigma \pi d^2/(4 \cdot l) \qquad (1)$$

The specific conductivity of the pore interior in a 1 M KCl solution is assumed to be 110 mS/cm. This means that the effective diameter of the high conductance pore ($\Lambda = 4.0$ nS) is about 1.7 nm, whereas that of the pore with the smaller conductance ($\Lambda = 2.4$ nS) is approx. 3 nm. Similar diameters have been estimated for most mitochondrial porins investigated to date [4–6,13,27,28]. Mitochondrial porins are voltage-dependent and switch

to ion-permeable 'closed' states [6]. The porin from *A. anguilla* is no exception, since single-channel experiments showed that both channels (the high- and low conductance pores) switch to substates at voltages larger than 20 mV. The voltage-dependence of fish porin was investigated with membranes in which many channels were reconstituted. Using the previously proposed formalism [5], we could show that approximately two gating charges were involved in the gating process. The midpoint potential of the channel distribution, i.e., the potential at which 50% of the total number of channels were in the 'closed' configuration was approx. 55 mV.

Selectivity of eel porin

The single-channel data suggested that porin of *A. anguilla* had a certain preference for anions over cations. To verify this, we measured the ion selectivity of porin by measuring the membrane potential under zero-current conditions. After the incorporation of about 100 to 1000 porin channels into the membranes, the salt concentration on one side of the membrane was raised 5-fold to 100 mM and the zero-current potential was measured 5–10 min after the gradient was established. The results are summarized in Table III. For 5-fold gradients of KCl and LiCl the more dilute side (20 mM) was always negative which indicated preferential movement of the anions through the porin channel in the open state, i.e., the channel is anionically selective for these two salts. However, the channel selectivity was not independent of the salt and the more dilute side became positive in the case of potassium acetate. This result indicated preferential movement of the cation over the anion for the combination of the mobile potassium cation and the less mobile acetate anion. Analysis of the data of Table III using the Goldman-Hodgkin-Katz equation [23] gave some information about the ratios of the permeabilities, P_a, for anions and P_c for cations. They ranged between 0.45 for KCH_3COO and 4.0 for LiCl. This result indicated that the aqueous mobility of the ions plays a certain role in channel selectivity, which is not the case for channel exclusively selective for anions or cations.

TABLE III

Zero-current membrane potentials, V_m, of membranes from diphytanoyl phosphatidylcholine/n-decane in the presence of porin of A. anguilla measured for a 5-fold gradient of different salts

V_m is defined as the difference between the potential on the dilute side (20 mM) and the potential at the concentrated side (100 mM). The pH of aqueous salt solutions was around 6 unless otherwise indicated; $T = 25°C$. P_a/P_c was calculated from the Goldman-Hodgkin-Katz equation from at least three individual experiments [23].

Salt	V_m (mV)	P_a/P_c
KCl	−10	1.8
LiCl	−22	4.0
KCH_3COO (pH 7)	15	0.40

Discussion

We have described the isolation and purification of a protein preparation from liver mitochondria of the fish *A. anguilla* using the same method devised for the isolation and purification of porin from whole mitochondria from mammalian tissues and primitive eukaryotic cells [6,12,16,27]. The protein preparation showed two bands with very similar molecular masses on SDS-electrophoretograms. Reconstituted into lipid bilayer membranes the protein fraction showed pore-forming activity with two main peaks of the single-channel conductance at 2.4 and 4.0 nS in a 1 M KCl solution. The large conductance peak, which corresponds to an estimated diameter of 1.7 nm, is common to all eukaryotic porins, with the sole exception of *Paramecium*, whose estimated diameter is only 1.3 nm (5,27; see also Table I). The smaller conductance peak cannot be simply considered half of the large channel. Moreover we have reported the presence of stable smaller single-channel conductances of mitochondrial porin [5,28]. We do not know at this time whether the smaller channel is a different configuration of mitochondrial porin or an artifact caused by the isolation and/or the reconstitution method. In any case we have clearly demonstrated in this study that the 2.4 nS channel formed by mitochondrial porin has besides the smaller single-channel conductance very similar properties than the 'normal' 4.0 nS channel, namely, it is also voltage-dependent and it is also anionically selective in the 'open' state.

The molecular analysis of the *A. anguilla* hydroxyapatite/cellulose eluate revealed the presence of two bands of very similar electrophoretic mobility (32 and 32.5 kDa) and variable intensities. Both bands cross-reacted with antibodies raised against bovine heart porin and against the 19-amino-acid long N-terminal end of human porin. The two bands were cut out from the gel and their peptide maps were obtained by digestion of the proteins with proteolytic enzymes. The peptide maps were very similar, but some differences were observed. Unfortunately, we were not able, either by changing the detergent [29] or by changing the chromatographic protocol (data not shown), to obtain in a native way the separation of the two bands. This would have been needed to demonstrate differences in pore-formation between the 32 kDa and the 32.5 kDa proteins.

On the basis of the molecular analysis we cannot exclude the presence of several pore-forming proteins in the mitochondrial outer membrane. These differences could consist of small changes within the primary se-

quence as shown here or as has been suggested previously [30]. On the other hand, the possible existence of other different pore-forming proteins in the mitochondrial outer membrane cannot be ruled out. In a recent paper [14], the disruption of the gene encoding for the only known pore-forming protein of yeast resulted in a mutant which was able, after some time of adaptation, to save the most important mitochondrial functions [31]. In fact a second general diffusion pore has been identified by reconstitution experiments using detergent solubilized mitochondrial outer membranes from a porin-free yeast mutant [15]. Its single-channel conductance is 0.21 nS in 0.1 M KCl and the estimated diameter of the pore 1.2 nm (Table I). The lack of additional protein bands induced in the polypeptide pattern of the outer membrane of the porin-free yeast mutant suggests that the second pore-forming protein is also present in wild-type strains [15]. This somewhat unexpected result implicates the existence of other permeability pathways which can mimic the very important role of porin in mitochondria, in analogy to the many porins of Gram-negative bacteria [2].

The immunological analysis revealed that the two bands present in eel porin cross-reacted with antibodies raised against bovine heart porin but not with antibodies against porin of *S. cerevisiae* or of *N. crassa*. It was surprising to find cross-reactivity of eel porin with antibodies raised against a synthetic polypeptide consisting of the 19-amino-acid long N-terminal end of human porin. This result clearly indicates extensive structural similarities between fish and mammalian porins. The N-terminal portion of eukaryotic porins seems to be a rather conserved part of the total protein. It forms an amphiphilic α-helical structure which is conserved among organisms as distantly related as fungi [9,10] and humans [11], even when the amino acid sequences do not show high homology. The amphiphilic α-helix at the N-terminus of the porins could be required for protein insertion into the outer membrane [10], which would explain the homology and, in turn, the immunological cross-reactivity between eel and mammalian porins.

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