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Minireview

Transmembrane Arrangement of Mitochondrial Porin or Voltage-Dependent Anion Channel (VDAC)

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Porin or voltage-dependent anion-selective channel (VDAC) is the main protein responsible for the high permeability of the outer mitochondrial membrane. The mitochondrial porin is mainly composed of sided β-strands, in analogy with bacterial porin, whose structure has been resolved at 1.8 Å resolution. In mitochondrial porins the N-terminal region forms an amphipathic α-helix, a structure conserved in organisms very distant from the evolutionary point of view. This part of the protein is exposed to the water phase, as demonstrated by ELISA assays. Various extramembranous loops have been identified by specific proteolytic cleavages. These overall, combined results were used to draw a model of the transmembrane arrangement of mammalian porin. This model is compared to other mitochondrial and bacterial porin models.

KEY WORDS: Porin; VDAC (voltage-dependent anion-selective channel); transmembrane arrangement; β-strands; mitochondria.

INTRODUCTION

The high permeability of the mitochondrial outer membrane is caused by the presence of a general diffusion pore called the voltage-dependent anion-selective channel (VDAC) or mitochondrial porin. Low anion selectivity has been demonstrated in lipid bilayer reconstitution studies, and molecules as large as 6–8 kDA have been shown to pass through the pore at applied voltages of 5–10 mV (Colombini, 1979, 1989; Benz, 1985; De Pinto *et al.*, 1987). The pore has been shown to be voltage-dependent, with the extent of this dependence varying among different tissues. Furthermore it has been shown that the hexokinase-binding protein present in the outer mitochondrial membrane is identical with the mitochondrial porin (Linden *et al.*, 1982b; Fiek *et al.*, 1982). The amino acid sequence of the mitochondrial porins from *Saccharomyces cerevisiae* (Mihara and Sato, 1985), *Neurospora crassa* (Kleene *et al.*, 1987), and human B-lymphocytes (Kayser *et al.*, 1989) has been determined. A great deal of interest is focussed nowadays on the structural arrangement of the polypeptide chain(s) of porin and its/their role in the properties of the channel. In this review we summarize and compare the most recent information about this topic.

PURIFICATION OF PORIN

Two rapid methods are now available for the purification of functional mitochondrial porin to homogeneity (for a review, see Palmieri and De Pinto, 1989). Both make use of a mixed hydroxyapatite/celite column as a single chromatographic step. One method uses the detergent Triton X-100 and the other the detergent lauryl (dimethyl)amine oxide (LDAO). With Triton X-100 porin passes through the column, whereas with LDAO it is first bound to the column and then eluted by the addition of salts. The reason for the different chromatographic behavior lies in the chemical structure of the detergents. Triton X-100 has

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a very large polar head group which shields the surface-exposed hydrophilic domains of the protein LDAO, on the other hand, has a very small polar head group which leaves part of the hydrophilic domains, including some positively charged groups of the protein, exposed to the surrounding water. These charged groups are responsible for the interaction of porin with the chromatographic material.

External charged groups of porin must be either totally or predominantly positively charged at neutral pH, since the LDAO-purified protein binds only to cation- and not to anion-exchangers (De Pinto *et al.*, 1989a). The interaction between the LDAO-porin and the cation-exchanger column is thus a suitable system to investigate the positively charged residues located on the external surface of the porin-detergent micelle functionally active upon reconstitution (Palmieri and De Pinto, 1989). By labelling LDAO-porin with fluorescein isothiocyanate (FITC), a fluorescent, hydrophilic reagent specific for amino groups, the binding of porin to the cation-exchanger was abolished (De Pinto *et al.*, 1990).

PROPERTIES OF PURIFIED PORINS

The purification procedures described above have been successfully applied by us to mitochondria derived from both primitive and advanced eukaryotic cells. In this way the properties of many different mitochondrial porins could be compared. In all mitochondria studied so far, the presence of a polypeptide of molecular mass ranging between 29 and 37 kDa in different species was shown (Freitag *et al.*, 1982; Linden *et al.*, 1982a; Colombini, 1989; De Pinto *et al.*, 1987, 1989b, 1991a). These polypeptides have been identified as porin on the basis of their functional properties, when reconstituted in black lipid membranes, of their peptide maps, and of their immunological cross-reactivity with antisera raised against other porins. The properties of different porin preparations are summarized in Table I.

In particular, the availability of antisera against different porins allowed us to perform an extensive comparison of the imunological similarities among eukaryotic porins. This immunological analysis is also shown in Table I. An increasing sensitivity to mammalian antisera (an antiserum to the purified bovine heart porin and an antiserum to the 18 N-terminal amino acids of human porin) was found as the evolutionary ladder was ascended. Interestingly, fish, echinoderma, and insect porins were detected by the antiserum directed against the human porin N-terminal end, indicating structural similarities in this region (Table I). The N-terminal region of porin forms an amphipathic α-helix. Although the primary sequence is not very similar, this amphipathic α-helix is preserved in organisms as distantly related as human and *S. cerevisiae* or *N. crassa* (Fig. 1). Two hypotheses presently exist about the role of the N-terminal sequence. Since porin has no cleavable pre-sequence, the N-terminal α-helix could be responsible for the import pathway of the protein (Kleene *et al.*, 1987). Alternatively, the N-terminal α-helix could be involved in the structural changes that porin undergoes during its partial closure (Mannella, 1990).

The 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).

SEQUENCE ANALYSIS AND STRUCTURAL PREDICTIONS OF PORINS

Bacterial porins consist predominantly of β-pleated sheet structure as demonstrated spectroscopically (Nabedryk *et al.*, 1988) and recently by crystal analysis (Weiss *et al.*, 1991).

None of the known mitochondrial porin sequences has a particularly hydrophobic primary structure (Mihara and Sato, 1985; Kleene *et al.*, 1987; Kayser *et al.*, 1989). According to predictions derived from the hydrophobic profiles, the secondary structure is composed of one, N-terminal, sided α-helix and several membrane-spanning sided β-sheets (Kleene *et al.*, 1987). A sided β-strand can be seen as a stretch of alternating hydrophobic residues, protruding into the interior of the bilayer, and hydrophilic ones protruding into the water-filled interior of the channel. This special structural feature of porin accounts for its properties as an integral membrane protein when solubilized, although its sequence is rather hydrophilic (47–49% of polar residues).

Twelve (Blachly-Dyson *et al.*, 1990), 15 (Kleene *et al.*, 1987), or 19 (Forte *et al.*, 1987) β-strands were predicted from the analysis of the yeast porin sequence. The number of β-strand segments crossing the membrane is very relevant. By postulating 15–19 transmembrane β-segments, indeed, a single porin

Table I. A Comparison of Eukaryotic Porins

Organism	Channel diameter[a]	Subunit M.W.[b]	Cross-reactivity with antisera against			
			BHP[c]	N-term	Yeast	*N. crassa*
Yeast						
S. cerevisiae	1.7	31 000	−	−	+++	+
Protozoa						
Paramecium tetraaurea	1.3	37 000	−	−	+	++
Insect						
Drosophila melanogaster	1.7	31 000	−	+	++	−
Echinoderma						
Paracentrotus lividus	1.7	34 000	++	++	−	N.D.
Fish						
Anguilla anguilla	1.7, 1.2	32 000	++	++	−	−
Mammalian						
Rattus norvegicus	1.7	35 500	+++	+++	−	−

[a]Determined in 1 M KCl as in Benz *et al.* (1978).
[b]Determined by SDS-PAGE.
[c]Abbreviations used: 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; N.D.: not determined. The + sign indicates presence of cross-reactivity, and the number of + signs indicates the intensity of the immunological reaction.

chain can be folded into a "β-barrel" type structure which has pore dimensions consistent with those determined by biophysical analysis (Colombini, 1979, 1989; Benz, 1985; De Pinto *et al.*, 1987) and electron microscopy studies (Mannella *et al.*, 1989). We carried out a refined analysis of the human porin sequence by looking for amphipathic secondary structures spanning the membrane. The graphic method for the analysis of amphipathic secondary structures (Link *et al.*, 1987) was developed on the basis of the Kyte and Doolittle (1982) work. With this method we found a number of 16 transmembrane β-strands to be the most probable arrangement of human porin (De Pinto *et al.*, 1991b).

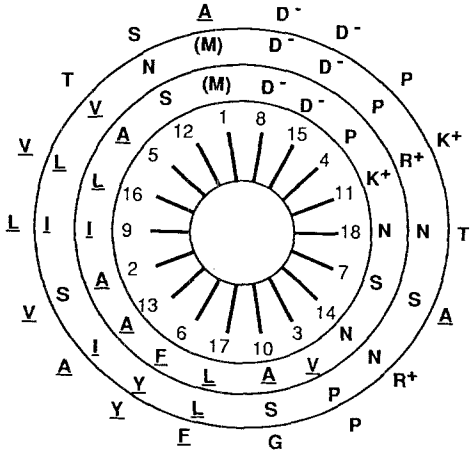

Fig. 1. Helical wheel projection of the amino-terminal region of porin. The first 18 amino acids of *N. crassa* (inner circle), of yeast (central circle), and of human porin (outer circle) are drawn in a helical wheel projection. Hydrophobic residues are underlined and charges are indicated. The numbers refer to the residue position in the sequence.

THE N-TERMINAL REGION OF MAMMALIAN PORIN IS EXPOSED TO THE WATER PHASE

The water-exposed localization of the N-terminus of porin has been demonstrated by enzyme-linked immunosorbent assays (ELISA) that used antisera raised against synthetic peptides with the human porin N-terminus sequence. The reaction of the antisera with porin in its native location in the mitochondrial outer membrane was detected by direct reaction of immobilized mitochondria with antibodies and by back-titration. This last experiment was devised to rule out the possibility that coating the mitochondria on the microtiter plates could disorganize the membrane structure (De Pinto *et al.*, 1991b).

Immunotitrations with anti-N-terminal antibodies were performed using intact or broken mitochondria coated onto ELISA plates. In the latter titrations, osmotic shock and freeze/thawing were used to break the mitochondrial membranes, making the inner as well as the outer side of the outer membrane accessible to antibodies. Both type of particles yielded the same results in ELISA assays, suggesting that the

N-terminal region of porin is located on the external and not on the internal side of the outer mitochondrial membrane.

PROTEOLYSIS OF MEMBRANE-BOUND MAMMALIAN PORIN REVEALED SPECIFIC CLEAVAGE SITES

In order to identify hydrophilic, extramembranous segments of porin and their orientation, the access of different proteases to the peptide chain of the membrane-bound protein was investigated using intact or broken bovine heart mitochondria. The fragments generated upon proteolysis were detected by immunoblotting using antisera raised against the bovin heart porin and the N-terminus of the human porin.

In a first set of experiments, we used carboxypeptidase A to check whether it was possible to shorten the polypeptide chain in intact or in broken mitochondria. The treatment with carboxypeptidase A did not increase either the electrophoretic migration of porin or the intensity of the immunodetected band (De Pinto *et al.*, 1991b). The lack of porin cleavage in the mitochondrial membrane by carboxypeptidase A has been interpreted by us to indicate that the C-terminus is not exposed to the water phase since it is not accessible to the enzyme. In line with an intramembranous location of the C-terminus is the fact that the analysis for prediction of antigenic determinants of human porin, based on the Hopp and Woods (1981) algorithm, has indicated a very low level of immunogenicity for the C-terminus. The relevance of the C-terminal region of yeast porin in the import of the newly synthesized protein into the mitochondrial outer membrane was stressed by Hamajima *et al.*, (1988). Upon deletion of the carboxy-terminal 62 amino acids of the yeast porin, the protein lost its capacity to be correctly imported into the mitochondria (Hamajima *et al.*, 1988).

In other experiments intact and broken mitochondria were subjected to endoproteolysis, looking for cleavage sites located in water-exposed domains of porin. Only a few of the proteases assayed were able to cleave porin, and the cleavage occurs in a very specific way, usually at only one site. Based on the number of the fragments generated upon enzymatic digestion of porin and their molecular weight, we proposed that there is one cleavage site for trypsin, located between residues 108 and 119 (where 5 lysines and 1 arginine are present), two cleavage sites for chymotrypsin at positions 117 and 172, and one for protease V8 at D_{227} or D_{229} (De Pinto *et al.*, 1991b). It should be stressed that porin was completely cleaved by protease V8, trypsin, and chymotrypsin in intact mitochondria. This suggests that most if not all the extramembranous domains of porin are exposed to the outside and all the porin molecules have the same orientation in the outer mitochondrial membrane, since in a different arrangement of the poplypeptides only part of the molecules would have been cleaved.

MODELS OF TRANSMEMBRANE ARRANGEMENT OF PORIN

Figure 2 shows a model of the transmembrane arrangement of the mammalian porin. It is based on the experiments described above. Most of the protein is embedded in the phospholipid bilayer. The amphipathic N-terminal α-helix is accessible to the water phase. Two major loops protrude into the external water phase. All large hydrophilic domains and all the cleavage sites are exposed to the outside of the mitochondrial outer membrane. The most important antigenic epitopes should be localized in these regions, since the C-terminal part of the protein seems poorly detectable by polyclonal antisera. All these results are compatible with a model where most if not all the extramembranous domains of porin are exposed to the outside and all the porin molecules have the same orientation.

On the basis of site-directed mutagenesis experiments, Blachly-Dyson *et al.*, (1990) proposed a model of the transmembrane arrangement of *S. cerevisiae* porin in which 12 β-strands and the amino-terminal α-helix cross the membrane. Blachly-Dyson *et al.*, (1990) identified 14 functionally important sites, distributed throughout the length of the molecule. Mutation at one or more of these 14 sites altered the selectivity of the channel for ions. This 12-strand model omits a large part of the sequence from the membrane since mutations in that range do not alter the channel behavior of the corresponding mutant proteins. Comparison with our model showed that the 14 important positions are also within the membrane in human porin, but 9 of the 14 residues are not conserved in the human porin although the channel properties seem to be identical. The conserved residues are D_{15}, K_{19}, D_{29}, K_{60}, and K_{95}.

It is interesting to compare our model with the

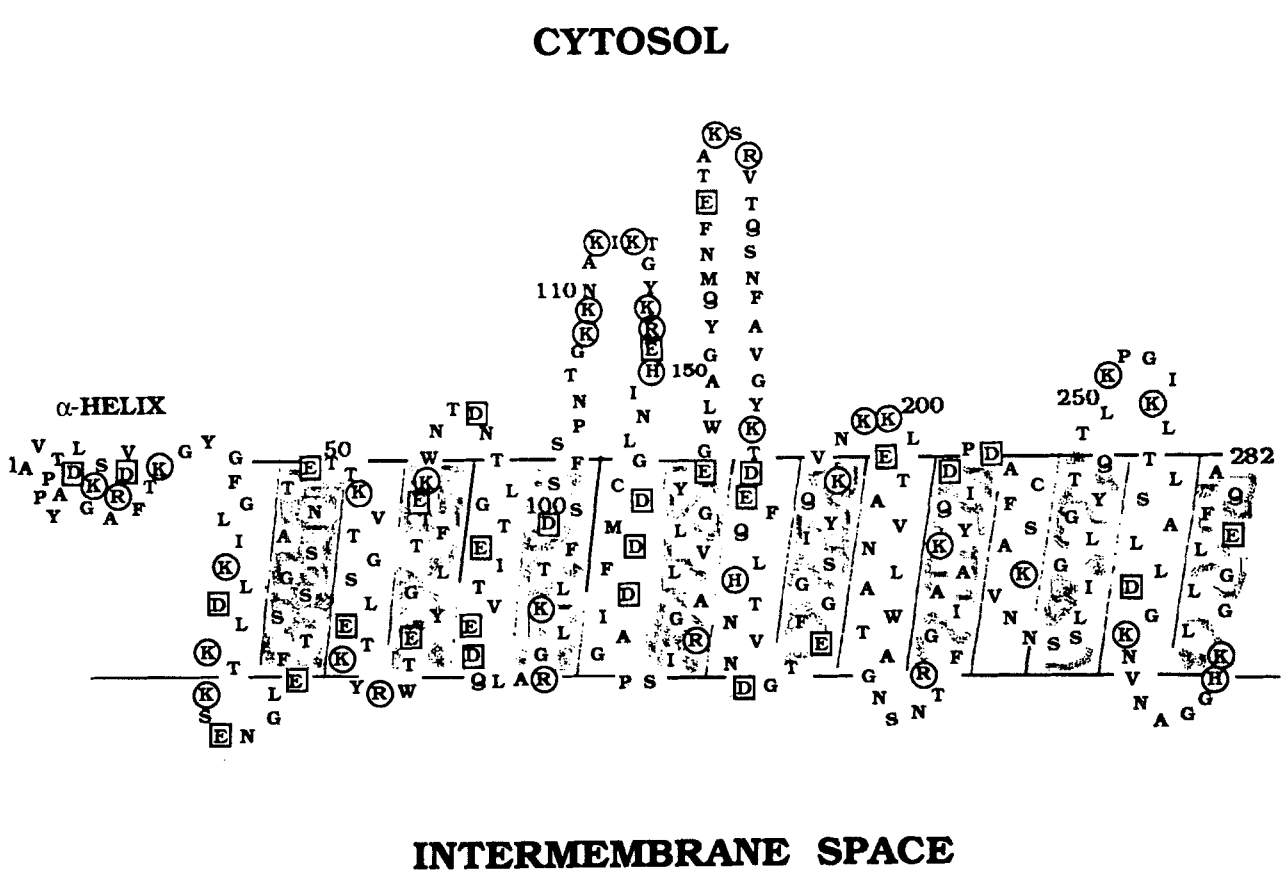

Fig. 2. Model of transmembrane arrangement of the polypeptide chain of human porin. A model drawn for the human B-lymphocyte porin sequence. The starting amphipathic α-helix lies on the external surface of the membrane. 16 amphipathic β-strands cross the membrane. Positively charged amino acids are indicated by circles, negatively charged by squares.

crystal structure of porin from *Rhodobacter capsulatus* which was determined at 1.8 Å resolution by Weiss *et al.*, (1991). They also found 16 β-strands crossing the membrane to form a "β-barrel" structure, which was large enough to form a channel. Two major loops are water-exposed protruding from the same side of the membrane. There is no trace, however, of an N-terminal amphipathic α-helix. The distribution of charged residues in *R. capsulatus* porin is remarkable. In one-half of the structure, divided at the barrel equator, there are as many as 44 negative and 12 positive charges, whereas in the other half there are only 7 positive and 7 negative charges. This electrostatic asymmetry should have a relevant effect on the functional properties of the pore (Weiss *et al.*, 1991). Also in our model an asymmetric distribution of charged residues can be observed. Eighteen positively and 11–12 negatively charged residues (including those of the amphipathic N-terminal α-helix) are exposed to the external water phase, while only 6–8 positive and 3 negative are exposed to the internal water phase.

While the basic orientation of mitochondrial porin in the membrane seems to be quite clear, it is not yet clear how many polypeptides are involved in forming a single channel unit. Recent results from electron microscopy of frozen-hydrated *N. crassa* outer mitochondrial membrane crystals favor a monomer channel as does STEM work of Thomas *et al.*, (1991). Mannella *et al.*, (1989) showed that a β-barrel consisting of a 3.8 nm-diameter alpha-carbon cylinder, with a 0.5 nm-thick "shell" of amino acid residues on either side, would be consistent with most of the known features of porin. The inner diameter of such a channel would be 2.8 nm, a value which is in agreement with those based on negative stain exclusion from the channel lumen and is in the range of diameters predicted by reconstitution experiments (Colombini, 1979, 1989; Benz, 1985; De Pinto *et al.*, 1987).

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