Peptide-Specific Antibodies and Proteases as Probes of the Transmembrane Topology of the Bovine Heart Mitochondrial Porin†

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ABSTRACT: We have investigated the transmembrane topology of the bovine heart mitochondrial porin by means of proteases and antibodies raised against the amino-terminal region of the protein. The antisera against the human N-terminus reacted with porin in Western blots of $NaDodSO_4$-solubilized bovine heart mitochondria and with the membrane-bound porin in enzyme-linked immunosorbent assay (ELISA). The immunoreaction with mitochondria coated on microtiter wells showed that the amino-terminal region of the protein is not embedded in the lipid bilayer but is exposed to the cytosol. Back-titration of unreacted anti-N-terminal antibodies after their incubation with intact mitochondria demonstrated that the porin N-terminus is also exposed in "noncoated" mitochondria. No difference in antisera reactivity was observed between intact and broken mitochondria. Intact and broken mitochondria were subjected to proteolysis by specific proteases. The membrane-bound bovine heart porin was strongly resistant to proteolysis, but a few specific cleavage sites were observed. *Staphylococcus aureus* V8 protease gave a large 24K N-terminal peptide, trypsin produced a 12K N-terminal and an 18K C-terminal peptide, and chymotrypsin gave two peptides of M_r 19.5K and 12.5K, which were both recognized by the antiserum against the human N-terminus. Carboxypeptidase A was ineffective in cleaving the membrane-bound porin in both intact and broken mitochondria. Thus, the carboxy-terminal part of the protein is probably not exposed to the water phase. The cleavage patterns of membrane-bound porin, obtained with *S. aureus* V8 protease, trypsin, and chymotrypsin, showed no difference between intact and broken mitochondria, thus indicating that all porin molecules have the same orientation in the membrane. The computer analysis of the sequence of human B-lymphocyte porin suggested that 16 β-strands can span the phospholipid bilayer. This result, together with the overall information presented, allowed us to draw a possible scheme of the transmembrane arrangement of mammalian mitochondrial porin.

The mitochondrial outer membrane shows special permeability properties in that it is freely permeable to hydrophilic substances up to a well-defined size of the hydrated molecules (Colombini, 1979; Benz, 1985). The free permeability of the mitochondrial outer membrane is caused by the presence of a general diffusion pore called the voltage-dependent anion-selective channel (VDAC)[1] or mitochondrial porin. All mitochondria studied so far show the presence of porin, a polypeptide of M_r ranging between 29K and 37K in different species (Freitag et al., 1982a; Linden et al., 1982; Colombini, 1989; De Pinto et al., 1987a, 1989b, 1991). Deletion of the gene encoding this polypeptide leads, after some adaptation, to the overexpression of other pore components with different functional features (Dihanich et al., 1989).

Two methods have been established for the purification of functional mitochondrial porin to homogeneity (Palmieri & De Pinto, 1989). One method uses the detergent Triton X-100 (De Pinto et al., 1987b) and the other lauryl dimethyl amino oxide (LDAO) (De Pinto et al., 1989a). In comparison with Triton X-100, LDAO has a much shorter polar head group. Porin purified in the presence of LDAO shows better preserved functional features (De Pinto et al., 1989a). Furthermore, LDAO-porin has part of its hydrophilic domains exposed to the water phase, since it is able to interact with cation exchangers (Palmieri & De Pinto, 1989; De Pinto et al., 1990).

Recent papers have provided valuable information about the structure of VDAC. The primary structures of porins from *Saccharomyces cerevisiae* (Mihara & Sato, 1985), *Neurospora crassa* (Kleene et al., 1987), and human B-lymphocytes (Kayser et al., 1989) have been determined. Porin from human B-lymphocytes shows approximately 29% and 24% identity to the sequences of porins of *N. crassa* and *S. cerevisiae*, respectively. In addition, immunological cross-reactivity in the N-terminal region of porin has been found among mitochondrial porins as distantly related as those from insect and human sources (De Pinto et al., 1991). Furthermore, there is over 90% identity between the human porin sequence and partial sequences of bovine heart and rat kidney mitochondrial porins (Kayser et al., 1989). Computer-based search for secondary structures suggested the only recurrent ones in *N. crassa* and *S. cerevisiae* porin to be sided β-strands (Kleene et al., 1987). The 18 amino-terminal residues should form

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[1] Abbreviations: Arg-C endoprotease, arginine-specific endoprotease; BSA, bovine serum albumin; DTE, dithioerythritol; ELISA, enzyme-linked immunosorbent assay; LDAO, lauryl dimethyl amino oxide; Lys-C endoprotease, lysine-specific endoprotease; PBS, phosphate-buffered saline consisting of 140 mM NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4; PBS-T, PBS supplemented with 0.05% (w/v) Tween 20; SPAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion-selective channel.

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an amphipathic α-helix in all three known sequences. Surprisingly, human porin was detected in the plasmalemma of B-lymphocytes by immunofluorescence experiments (Thinnes et al., 1989). It is not yet clear why the mitochondrial outer membrane pore-forming polypeptide is inserted in the plasmalemma of these cells.

On the basis of site-directed mutagenesis experiments, Blachly-Dyson et al. (1990) have 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. In this paper we have used specific proteases and antibodies, raised against the amino-terminal part of the human B-lymphocyte porin, to investigate the topology of porin in the bovine heart outer mitochondrial membrane. The results of these studies, together with a computer search aimed at detecting the putative sided β-strands in the mammalian sequence, have allowed us to draw a model of the transmembrane arrangement of the mammalian porin.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad and lauryl dimethyl amino oxide (LDAO) from Serva. Celite 535 was purchased from Roth, *o*-phenylenediamine dihydrochloride from Aldrich, and Tween 20 from Sigma. *Staphylococcus aureus* proteinase V8 was from Miles Laboratories. Carboxypeptidase A, Arg-C- and Lys-C-specific endoproteases, and chymotrypsin were from Boehringer, and trypsin–TPCK was from Merck.

Biological Preparations. Bovine heart mitochondria were prepared according to the method of Smith (1967). For antisera assays, we used freshly prepared intact bovine heart mitochondria, diluted at the appropriate concentrations in 120 mM KCl, 10 mM Tris, and 1 mM EDTA, final pH 7.2. Alternatively, the mitochondria, diluted in 12 mM KCl, 10 mM Tris, and 1 mM EDTA, final pH 7.2, were subjected to three cycles of freezing and thawing. This procedure breaks the mitochondrial membranes and makes the internal side of the outer membrane accessible to antibodies.

The M_r 20K peptide was obtained after treatment with CNBr of the purified bovine heart porin. The peptides were separated by SPAGE and electroeluted as described by Hunkapiller et al. (1983). The electroeluted peptide was chromatographed on Sephacryl S-200 (Pharmacia) in 70% formic acid and lyophilized.

Purification of Porin. The purification of bovine heart porin in LDAO was performed as in De Pinto et al. (1989b). Mitochondrial membranes (5 mg of protein/mL) were solubilized for 30 min with 2% LDAO, 10 mM Tris-HCl, pH 7.0, and 1 mM EDTA. The solubilization mixture was centrifuged at 40000*g* for 30 min, and the supernatant was applied onto a well-packed, dry, hydroxyapatite/Celite (ratio 2:1) column. After it had absorbed the solubilized material, the column was washed with the solubilization buffer. Then, the column was eluted with the solubilization buffer supplemented with 5 mM KP_i and 50 mM KCl (pH 7.0). Several fractions were collected and analyzed by SPAGE. The fractions containing pure porin were used for further experiments.

$NaDodSO_4$ Gel Electrophoresis. Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% $NaDodSO_4$. The separation gel contained, routinely, 14% acrylamide with a ratio acrylamide/bis(acrylamide) of 30:0.8 (Laemmli, 1970). When indicated, the discontinuous Tricine system of Schägger and von Jagow (1987) was employed. We used 15.5% acrylamide with a ratio bis(acrylamide)/acrylamide of 15.5:1, with 10% glycerol included in the separation gel. The molecular weight markers used were the Bio-Rad low molecular weight markers (92.5K, 66.2K, 45K, 31K, 21.5K, and 14.4K) and the MW-SDS-17 kit from Sigma, containing CNBr fragments of myoglobin [17K, 14.4K, 10.7K (fragment "1 + 3", always found in the peptide mixture; Schägger & von Jagow, 1987), 8.2K, 6.2K, and 2.5K).

Immunoblotting Experiments. The 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 in rabbit after 3 and 5 weeks, respectively. Blood was collected from the marginal ear vein. The other three antisera employed were raised against the whole porin purified from human B-lymphocytes; against a synthetic peptide mimicking the first 19 residues of porin from human B-lymphocytes, but with the amino terminal free; and against the first 19 residues of porin purified from human B-lymphocytes with the amino terminal acetylated, as found in the purified protein (Kayser et al., 1989). This last antiserum, referred to in this paper as anti-N-terminus, was used in all the experiments shown unless otherwise specified. Preparation and characterization of these antibodies are detailed in Thinnes et al. (1989).

The proteins separated on a SPAGE were transferred to nitrocellulose (Rott & Nelson, 1981; Towbin et al., 1979) and incubated with the antisera and then with an anti-rabbit Ig horseradish peroxidase linked antibody, purchased from Amersham. The peroxidase reaction was performed using 20 mL of a mixture of 0.05% 4-chloro-1-naphthol, 16% methanol, and 0.5% BSA in 0.14 M NaCl and 0.01 M phosphate (pH 7.0) with the final addition of 12 μL of 30% H_2O_2.

Antibody Assays by ELISA. The ability of antisera to react with membrane-bound porin was tested by ELISA using microtitration poly(vinyl chloride) plates (Titertek, catalog no. 77-172—high activated, lot bo-14-n3, from Flow Laboratories). Freshly prepared intact or broken bovine heart mitochondria, diluted at the appropriate concentrations, were coated on plate wells overnight at 4 °C under gentle shaking (100 μL per microtiter plate well). After coating, the plates were washed twice with PBS-T and then incubated 1 h with 1% BSA in PBS in order to saturate the unspecific sites. After two washes with PBS-T, 100 μL of antiserum in PBS-T was added to the wells and incubated for 2 h at room temperature. The plates were washed five times with PBS-T, and 100 μL of peroxidase-conjugated anti-rabbit Ig, diluted 2000-fold, was added to each well. This incubation also lasted 2 h at room temperature under shaking. After five more washes, 100 μL of 0.4 mg/mL *o*-phenylenediamine and 0.03% H_2O_2 dissolved in 0.1 M citrate phosphate buffer, pH 5.0, was added. The peroxidase reaction was left to develop for $^1/_2$ h at room temperature in the dark and was then stopped by the addition of 25 μL of 4 M H_2SO_4. The absorbance was determined at 450 nm using an automatic reader (Titertek Multiskan PLUS MK II, Flow Laboratories).

Proteolytic Digestions. Intact and broken mitochondria were used for proteolytic digestion of membrane-bound porin. Intact mitochondria were diluted at a concentration of 10 mg/mL mitochondrial protein in a buffer containing 250 mM sucrose, 10 mM Tris, and 1 mM EDTA at a final pH of 7.4. Broken mitochondria were diluted in the same buffer or in a buffer having a tenth of the sucrose concentration. Samples (100 μL) of these mixtures were incubated with the enzymes indicated in the legends to figures and under Results section for 30 min and at a temperature of 37 °C. In the experiments with carboxypeptidase A, the buffer did not contain EDTA, which inhibits the enzyme, and its pH was raised to 8. At the

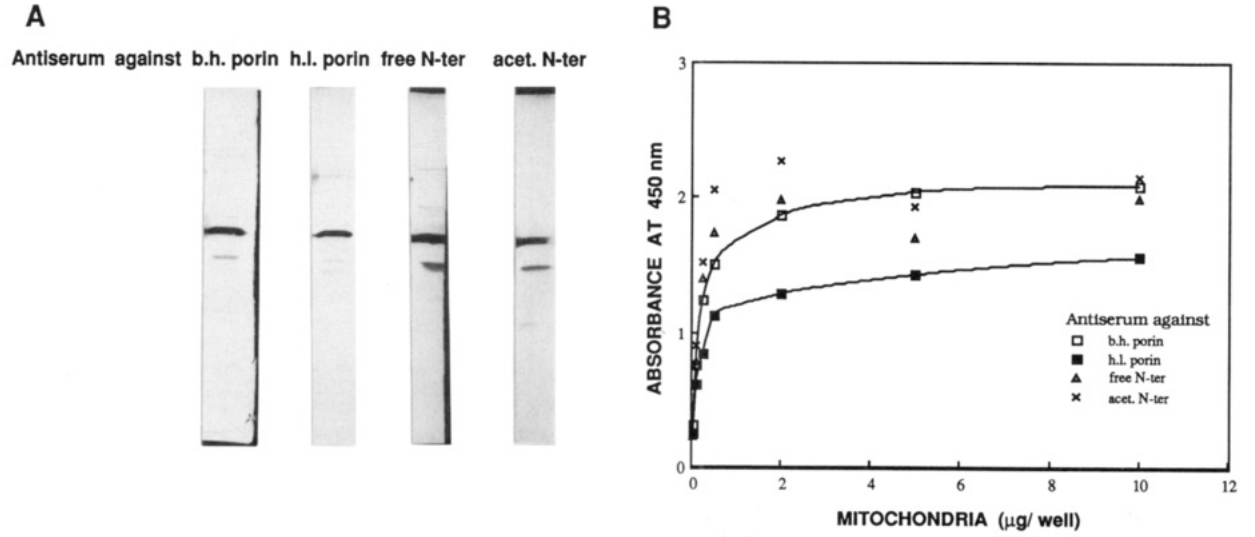

FIGURE 1: (A) Reactivity of different antisera toward the bovine heart mitochondrial porin assayed by Western blots. Aliquots of 100 μg of mitochondrial protein, dissolved in $NaDodSO_4$ sample buffer, were subjected to SPAGE and blotted on nitrocellulose. The nitrocellulose strips were immunodecorated with different primary antisera 500-fold diluted and anti-rabbit Ig horseradish peroxidase linked antibody. The primary antisera used were as follows, from left to right: b.h. porin, antiserum raised against purified bovine heart porin; h.l. porin, antiserum against purified human B-lymphocyte porin; free N-ter, antiserum against a synthetic peptide mimicking the 19 N-terminal residues of human B-lymphocyte porin; and acet. N-ter, antiserum against a synthetic peptide mimicking the 19 N-terminal residues of human B-lymphocytes porin with the N-terminus acetylated. (B) Reactivity of different antisera toward the membrane-bound porin in mitochondria assessed by ELISA. The wells of microtiter plates were coated with increasing concentrations of intact bovine heart mitochondria. The immobilized mitochondria were incubated with the different antisera diluted 400-fold, and the immunological reaction was detected as described under Materials and Methods.

end of the incubation, the reactions were stopped by adding 50 μL of a $NaDodSO_4$ buffer [225 mM Tris-HCl, pH 6.8, 7% $NaDodSO_4$ (w/v), 45% glycerol (w/v), 50 mM DTE, and traces of bromophenol blue] and by heating for 10 min at 100 °C. Then, aliquots of 100 μg of mitochondrial proteins were loaded on slab SPAGE and analyzed by immunoblotting. In control experiments we checked whether the action of the proteases used in this study (*S. aureus* protease V8, trypsin, and chymotrypsin) was effectively blocked by the "stopping" procedure. In these experiments proteolysis was blocked by the same procedure described above ($NaDodSO_4$ and boiling) 5 s after the addition of each protease to the mitochondrial suspension; then, the whole mixture was left to incubate for 30 min at 37 °C. Under these conditions porin was not cleaved at all by the proteases, as evidenced by the absence of fragments detected with either the anti-N-terminal antiserum or the anti-porin antiserum. It should be stressed that for effective block of the protease action both $NaDodSO_4$ and boiling were required. In the case of trypsin and chymotrypsin digestion another control has been performed; i.e., the reaction was blocked by the addition of the soybean trypsin inhibitor (Boehringer). No difference was observed in the proteolytic pattern obtained by this method as compared to the stopping procedure described above. The same pattern of immunodetected peptides generated from porin by the action of protease V8, trypsin, and chymotrypsin was obtained when the samples were precipitated by 10% trichloroacetic acid. In this case the pellets were washed first in 50% acetone (v/v) and 10% trichloroacetic acid, and then in acetone before being solubilized in $NaDodSO_4$.

Purified LDAO-porin was digested by *S. aureus* V8 protease, which was simply added to the protein solution and incubated at 37 °C for the indicated times. The reaction was stopped by heating for 10 min at 100 °C. The peptides were recovered by acetone precipitation, at a ratio of 5:1 of acetone:solution, overnight at −20 °C.

Structure Prediction Methods. Hydropathy plots were made as by Kyte and Doolittle (1982), whose hydropathy indices were used throughout. On the basis of the Kyte and Doolittle work we developed our own graphic method for the analysis of amphipathic secondary structures (Link et al., 1987). All programs were written in BASIC or FORTRAN and run on a Kontron PSI 80 microcomputer linked to a Graphtec MP 1000 plotter. In the weighted average plot, hydrophobic domains were detected, while amphipathic segments appeared to be neither hydrophobic nor hydrophilic. Possible sided β-sheets were detected in the sided β-sheet profile by alternating between hydrophobic and hydrophilic values every second residue. Possible amphipathic α-helices showed an alternation every third or fourth residue.

Other Methods. Mitochondrial protein was measured by the biuret method, using KCN to account for turbidity due to phospholipids (Kroger & Klingenberg, 1966). Purified protein was measured by the Lowry method modified for the presence of detergent (Kusov & Kalinchuk, 1978).

Results

Reactivity of Bovine Heart Porin toward Antibodies Raised against the N-Terminal End of Human Porin. Kayser et al. (1989) have recently sequenced the human B-lymphocyte porin. They found over 90% identity with partial sequences we had earlier obtained from bovine heart and rat kidney mitochondrial porins (Kayser et al., 1989). This large identity convinced us to use antisera raised against synthetic peptides, mimicking the amino-terminal part of human porin, for topological analysis of the bovine heart porin. Figure 1 shows a comparison of the reactivity of different antisera toward the bovine heart porin. Four antisera were used: the first raised against the whole porin purified from bovine heart mitochondria; the second against the whole porin purified from human B-lymphocytes; the third against a synthetic peptide mimicking the first 19 residues of porin from human B-lymphocytes, but with the amino terminal free; and the fourth against the first 19 residues of porin from human B-lymphocytes with the amino terminal acetylated, as found in the purified protein (Kayser et al., 1989).

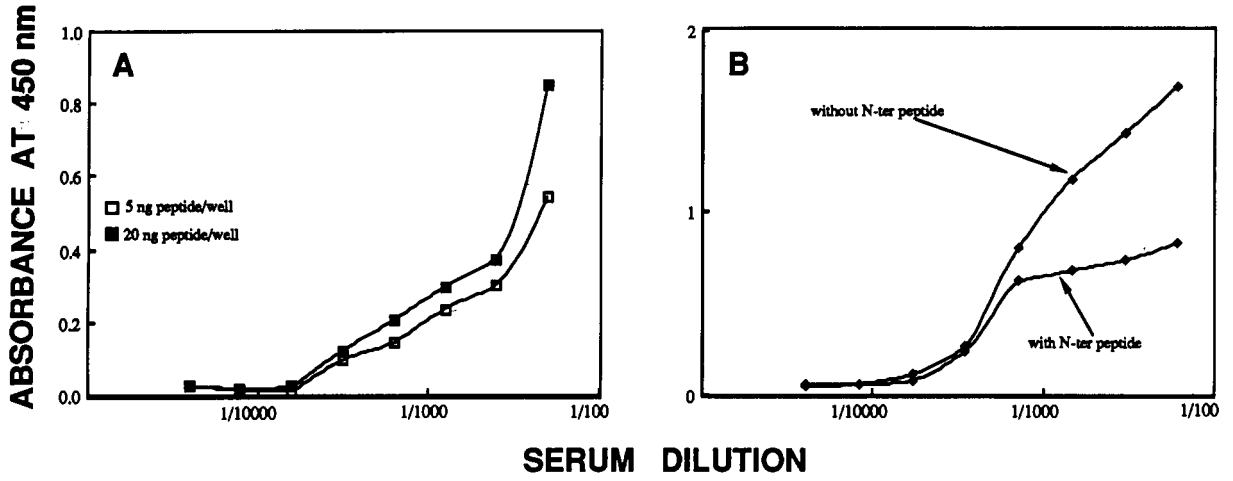

FIGURE 2: (A) Reactivity of the antiserum raised against the human B-lymphocyte porin N-terminus toward the N-terminal portion of bovine heart mitochondrial porin. The wells of microtiter plates were coated with 5 or 20 ng of the N-terminal CNBr peptide obtained from porin. The immobilized peptide was incubated with the anti-N-terminal peptide antiserum added at the indicated dilutions. Binding of antibodies was detected by a chromogenic reaction. (B) Inhibition of reactivity of the anti-N-terminal peptide antiserum toward the membrane-bound porin by the N-terminal CNBr peptide obtained from porin. The wells of microtiter plates were coated with 5 μg of intact mitochondria. The indicated dilutions of the antiserum against the porin N-terminus were preincubated (30 min at room temperature) in the presence or absence of 200 ng/mL N-terminal CNBr peptide from porin. Then, the immobilized mitochondria were incubated with the antiserum pretreated as above. Binding of antibodies was detected by a chromogenic reaction.

The Western blots of $NaDodSO_4$ lysates of bovine heart mitochondria, presented in panel A of Figure 1, demonstrated that all four antisera recognized a band of apparent M_r 35K corresponding to the M_r of the purified mammalian porins estimated using SPAGE (De Pinto et al., 1987a). In addition, the four antisera reacted, although to different extents, with a band of M_r 29–30K. It should be pointed out that a microheterogeneity in the electrophoretic behavior of purified porins from different sources has been shown by various authors (Linden et al., 1982; Thinnes et al., 1989). The possibility, however, that the lower band is a proteolytic product of porin cannot be excluded.

The reactivity of the four antisera toward the membrane-bound porin was investigated by ELISA with intact mitochondria coated on the wells of microtiter plates. With all the antisera, which were tested at the same dilution, the binding of the antibodies to mitochondria increased with the amount of mitochondria to a plateau value (Figure 1B). All the antisera, therefore, were able to detect the membrane-bound porin in situ. This means that some epitopes are recognized by the antisera in the physiological transmembrane conformation of porin. These epitopes must be water-exposed and localized on the external side of the outer mitochondrial membrane, since the diameter of the porin pore is too small to account for an immunological reaction with hydrophilic domains inside the pore. Among the different antisera tested, the antiserum against porin purified from bovine heart was more active than the antiserum against porin purified from human B-lymphocytes. Furthermore, the two antisera against the N-terminal end of the human porin, especially that against the acetylated N-terminus, appeared to be more active than the antiserum against the whole protein from bovine heart. The immunoreaction of the anti-N-terminal antibodies with intact mitochondria (Figure 1B) indicates that the N-terminal region of porin protrudes onto the cytosolic surface of the outer mitochondrial membrane.

Figure 2A shows that the antiserum against the acetylated N-terminus of human porin strongly reacted with the N-terminal CNBr peptide of bovine heart mitochondrial porin. The peptide, obtained from purified bovine heart porin, is a large fragment of M_r 20K, and its N-terminus is blocked as in the intact protein. It was coated on microtiter wells, and its reactivity was studied by ELISA. The data reported in Figure 2A show that amounts of peptide in the range of nanograms were detected by the antiserum against the acetylated N-terminus of human porin. In contrast, when the antiserum was assayed against the coated C-terminal CNBr peptide, no cross-reaction was detected (data not shown).

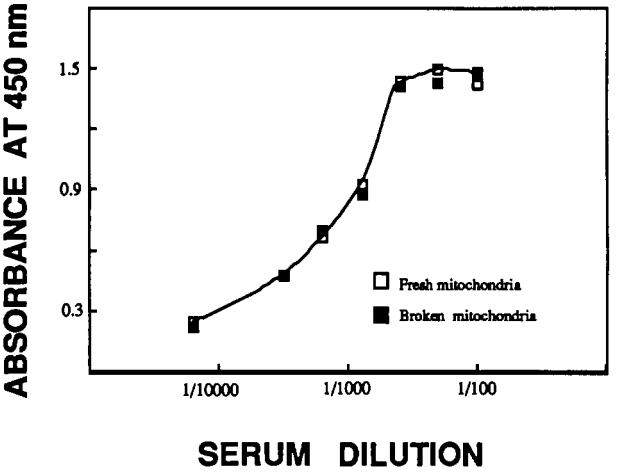

FIGURE 3: Reactivity of the anti-N-terminal antiserum toward the membrane-bound porin in intact and broken mitochondria, assessed by ELISA. Intact or broken mitochondria were coated on microtiter wells (5 μg of mitochondrial protein/well). The anti-N-terminal antiserum was added at the indicated dilutions, and the reactivity was assayed as described under Materials and Methods.

The specificity of the immunoreaction of the anti-N-terminal antibodies with intact mitochondria was investigated by adding the N-terminal CNBr peptide to the anti-N-terminal antiserum prior to the incubation with the coated mitochondria. The results of Figure 2B show that the reaction was drastically reduced when the antiserum was preincubated with 200 ng of N-terminal CNBr peptide.

Additional immunotitrations with anti-N-terminal antibodies were performed using coated intact or broken mitochondria. In the latter particles, osmotic shock and freeze/thawing were used to break the mitochondrial membranes, making the inner side of the outer membrane accessible to antibodies. The data presented in Figure 3 show that both types of particles yielded similar results, suggesting that the

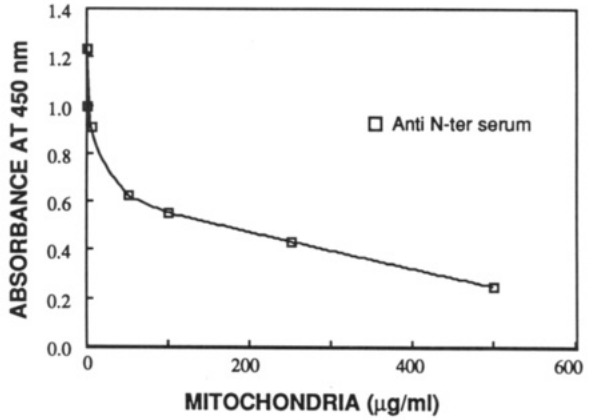

FIGURE 4: Back-titration by ELISA of unreacted anti-N-terminal antibodies after incubation with mitochondria. The anti-N-terminal antiserum diluted 800-fold was incubated with increasing concentrations of intact mitochondria. After 2 h, the mitochondria were sedimented by centrifugation and the unreacted antibodies in the supernatant were assayed by ELISA. The wells of microtiter plates were coated with 10 ng/well CNBr N-terminal peptide. Bound antibodies were determined as described under Materials and Methods.

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

It could be argued that coating the mitochondria on the microtiter plates could disorganize the membrane structure, resulting in access of anti-N-terminal antibodies to initially nonexposed epitopes of the membrane-bound porin. The following experiment was devised to test this possibility. Increasing concentrations of intact mitochondria were incubated with a fixed concentration of anti-N-terminal antiserum. After centrifugation the unreacted antibodies remaining in the supernatant were back-titrated by ELISA, using microtiter plates coated with the N-terminal CNBr peptide of bovine heart porin. The results of back-titration ELISA are shown in Figure 4. The reaction of the anti-N-terminal antibodies against the coated N-terminal peptide markedly decreased on increasing the amount of mitochondria, proving that "noncoated" mitochondria are able to bind the anti-N-terminal antibodies.

Enzymatic Digestion of Porin in Mitochondria. 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. In these experiments the fragments generated upon proteolysis were detected by immunoblotting.

In the experiment reported in Figure 5, intact mitochondria were incubated with *S. aureus* V8 protease at a ratio of 1:50 or 1:20 (protease:mitochondrial protein) for 30 min at 37 °C. After this incubation period the mitochondria were solubilized in $NaDodSO_4$ and the proteins were separated on slab gels and immunodecorated with the anti-porin or the anti-N-terminal antiserum. Both antisera clearly detected only one large peptide, the M_r of which was estimated to be 22K in the 14% Laemmli gel (Figure 5A) and 24K in the Schägger and von Jagow gel (Figure 5B). Proteinase V8 specifically cleaves the peptide bond at the carboxyl side of Glu at pH 4.0 and at the carboxyl side of both Glu and Asp at pH 8.0 (Houmard & Drapeau, 1972). We therefore repeated the same experiment in the presence of different buffers and pHs. The results of Figure 5B show that a 24K peptide was obtained after treatment of the mitochondria with proteinase V8 in Tris-HCl, pH 8 (lane 1), or in NH_4HCO_3, pH 8.2 (lane 3), but not in NH_4CH_3COO, pH 4.0 (lane 2). This indicates that the cleavage site of the membrane-bound porin by proteinase V8

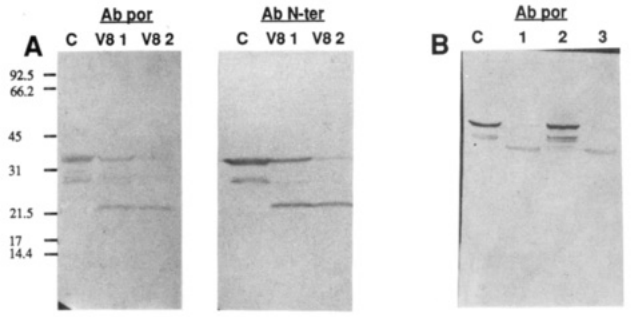

FIGURE 5: Cleavage of the membrane-bound porin by *S. aureus* protease V8. (A) Mitochondria resuspended in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EGTA were incubated without or with protease V8 at 37 °C for 30 min. Equal amounts of mitochondrial protein were run on 14% Laemmli gels, blotted on nitrocellulose, and immunodetected by antisera diluted 500-fold. On the left of the first panel, the electrophoretic migration in 14% Laemmli gel of the marker proteins with the indicated M_r's is reported. "Ab por" indicates that immunodetection of protein blotted on nitrocellulose was performed by means of an antiserum against the whole bovine heart porin. "Ab N-ter" indicates that the antiserum against the N-terminus of porin was used. Lanes C: Mitochondria incubated at 37 °C without proteases. Lanes V8 1: Mitochondria incubated with protease V8 at a ratio of 1:50 protease:mitochondrial protein. Lanes V8 2: Ratio 1:20 protease:mitochondrial protein. (B) Broken mitochondria resuspended in different buffers were incubated without or with protease V8 at 37 °C for 30 min. Equal amounts of mitochondrial protein were run on a Schägger and von Jagow gel, blotted on nitrocellulose, and immunodetected by the antiserum against the whole bovine heart porin (Ab por) diluted 500-fold. Lane C: broken mitochondria resuspended in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EGTA and incubated in the absence of protease at 37 °C for 30 min. Lane 1: Broken mitochondria resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EGTA and incubated at 37 °C for 30 min in the presence of protease V8 at a ratio of 1:50 (protease:mitochondrial protein). Lane 2: Broken mitochondria resuspended in 50 mM NH_4CH_3COO, pH 4.0, and 1 mM EGTA and incubated as above. Lane 3: Broken mitochondria resuspended in 125 mM sucrose, 50 mM NH_4HCO_3, pH 8.2, and 1 mM EGTA and incubated as above.

is at the level of an aspartic acid and that this cleavage site is accessible from the cytosolic side of the outer mitochondrial membrane.

In Figure 6 the effect of other proteases was studied in intact mitochondria. In panel A trypsin and chymotrypsin were used. The mitochondrial porin was efficiently cleaved by trypsin, which generated two large fragments with estimated M_r's of 18K and 12K, respectively. Both fragments were detected by the anti-porin antiserum, whereas only the smallest was identified by the anti-N-terminal antiserum. Therefore, it appears that one or more close cleavage sites for trypsin are located on the cytosolic surface of the outer mitochondrial membrane. Interestingly, this cleavage site is specific for mammalian porins, because porin from fungi was found to be resistant to trypsin in *N. crassa* mitochondria (Mihara et al., 1982; Freitag et al., 1982b). We tried to clarify whether Lys or Arg is present at the trypsin cleavage site of bovine heart porin. For this purpose we used the Arg-C- and Lys-C-specific endoproteases, but both were ineffective in cutting porin in mitochondria, even at very high concentrations (not shown). Like trypsin, chymotrypsin also cleaved the membrane-bound porin efficiently (Figure 6 A, lane Ch2). However, in this case two fragments (M_r 19.5K and 12.5K, respectively) were detected by both the anti-porin and the anti-N-terminal antiserum. These results suggest the existence of two chymotrypsin cleavage sites in the polypeptide chain of porin and that these sites are both accessible from the cytosolic side of the outer mitochondrial membrane. Porin was completely cleaved by all the effective proteases in both intact and broken

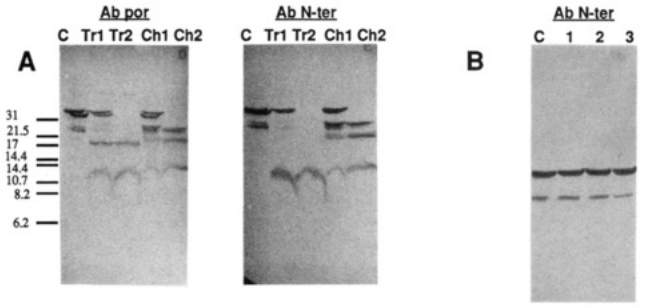

FIGURE 6: Cleavage of the membrane-bound porin by trypsin, chymotrypsin, and carboxypeptidase A. (A) Mitochondria resuspended in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EGTA were incubated without or with proteases at 37 °C for 30 min. Equal amounts of mitochondrial protein were run on Schägger and von Jagow gels, blotted on nitrocellulose, and immunodetected by antisera diluted 500-fold. On the left of the first panel, the electrophoretic migration in the Schägger and von Jagow gel of the marker proteins with the indicated M_r's is reported. "Ab por" indicates that immunodetection of protein blotted on nitrocellulose was performed by means of an antiserum against the whole bovine heart porin. "Ab N-ter" indicates that the antiserum against the N-terminus of porin was used. Lanes C: Mitochondria incubated at 37 °C without proteases. Lanes Tr1: Mitochondria incubated with trypsin at a ratio of 1:200 protease: mitochondrial protein. Lanes Tr2: Ratio 1:20 trypsin:mitochondrial protein. Lanes Ch1: Mitochondria incubated with chymotrypsin at a ratio of 1:200 protease:mitochondrial protein. Lanes Ch2: Ratio 1:20 chymotrypsin:mitochondrial protein. (B) Mitochondria in 250 mM sucrose and 10 mM Tris-HCl, pH 8.0, were incubated in the presence of increasing amounts of carboxypeptidase A at 37 °C for 30 min. Equal amounts of protein were run on a 14% Laemmli gel, blotted on nitrocellulose, and immunodetected by the antiserum against the N-terminus of porin (Ab N-ter) diluted 500-fold. Lane C: Mitochondria incubated without carboxypeptidase A. Lane 1: Mitochondria incubated with carboxypeptidase at a protease to mitochondrial protein ratio of 1:200. Lane 2: Ratio 1:50 protease:mitochondrial protein. Lane 3: Ratio 1:15 protease:mitochondrial protein.

mitochondria, as shown for protease V8 in panels A and B of Figure 5.

It is interesting to know whether or not the carboxy-terminal part of porin is exposed to water. Since the first Lys is 10 residues from the C-terminal amino acid, we used carboxypeptidase A to shorten the polypeptide chain of membrane-bound porin. Figure 6B shows that when intact mitochondria were treated with increasing concentrations of carboxypeptidase A, the electrophoretic migration of porin was not increased. The same result was obtained by treating broken mitochondria with carboxypeptidase A. This means that the carboxy terminus of porin is not accessible to the enzyme and therefore it is probably not exposed to the water phase.

Enzymatic Digestion of Porin Solubilized in LDAO. We have recently purified porin in LDAO and have demonstrated that this detergent is milder than Triton with respect to the activity of the voltage-dependent anion-selective channel (De Pinto et al., 1989b). It has been suggested that the membrane-bound porin exposes to the aqueous phase the same hydrophilic domains that the LDAO-porin micelle exposes to water (Palmieri & De Pinto, 1989). We therefore decided to try the same proteolytic cleavages performed with mitochondria onto porin in LDAO. Figure 7 shows the cleavage of LDAO-porin by *S. aureus* V8 protease. It was performed by adding the protease to the porin solution, without any other change which could be harmful to the porin conformation. A reproducible, well-defined peptide pattern was obtained. Five main polypeptides were visible with estimated M_r's of 28K, 24K, 15K, 11.5K, and 6.5K (Figure 7). Western blots of cleaved LDAO-porin performed with the anti-N-terminal antiserum showed two bands, corresponding to the 24K peptide

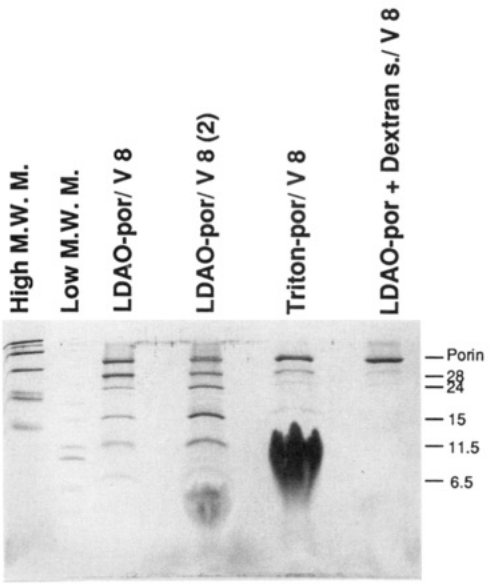

FIGURE 7: Cleavage of purified porin by *S. aureus* V8 protease. High M.W.M.: Bio-Rad low molecular weight markers (92.5K, 66.2K, 45K, 31K, 21.5K, and 14.4K). Low M.W.M.: MW-SDS-17 kit from Sigma (17K, 14.4K, 10.7K, 8.2K, 6.2K, and 2.5K). LDAO-por/V 8: Porin purified in LDAO (5 μg) was incubated with 1 μg of *S. aureus* V8 protease for 1 h at 37 °C and pH 7.0. The protease was inactivated by heating at 100 °C for 10 min. After acetone precipitation, the pellet was solubilized and run on a Schägger gel. LDAO-por/V 8 (2): LDAO-purified porin (5 μg) was precipitated in acetone. Then it was redissolved in 5 μL of 2% LDAO, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and incubated with 1 μg of V8 protease. Other conditions as described for the previous lane. Triton-por/V 8: Triton X-100 purified porin (5 μg) was incubated with 1 μg of V8 protease for 1 h at 37 °C. Other conditions as described for lane "LDAO-por/V 8". LDAO-por + Dextran s./V 8: LDAO-purified porin (5 μg) was preincubated with 2 mM dextran sulfate 5000 for 20 min, and then 1 μg of V8 protease was added. Other conditions as described for lane "LDAO-por/V 8". The large spot of low M_r in lane "LDAO-por/V 8 (2)" is mainly detergent [the protein purified in LDAO is largely delipidated; see De Pinto et al. (1989)]. The large spot in lane "Triton-por/V 8" may be due to both detergent and phospholipids. On the right side of the gel the M_r's of porin peptides are shown.

and to a small peptide in the 5–7K region not clearly stained by Coomassie blue (data not shown). The 24K V8 peptide comigrated with the 24K peptide obtained with the protease V8 treatment of the membrane-bound porin. The 15K peptide had the starting sequence (E_{49})-TTKV and the 11.5K (E_{176})-FQLH. The LDAO-porin, thus, has two additional V8 cleavage sites with respect to the membrane-bound protein. They are E_{49} and E_{176}. This demonstrates that few hydrophilic domains of porin are water-exposed, even in the protein–detergent micelle. In similar experiments performed on Triton-porin (Figure 7), and LDAO-porin preincubated with dextran sulfate (a polyanion known to modify the voltage dependence of the channel by interacting with surface positive charges) (Colombini, 1989; De Pinto et al., 1990) (Figure 7), we observed virtually no cleavage. In control experiments it was checked that neither 3% Triton X-100 nor 2 mM dextran sulfate affected the intrinsic activity of the protease V8 as tested on bovine serum albumin. The water-exposed domains of porin can thus be shielded by the large hydrophilic head group of the detergent Triton X-100 (Palmieri & De Pinto, 1989) or by other molecules (De Pinto et al., 1990). With trypsin and chymotrypsin the cleavage of the LDAO-porin was too drastic, and only a cloud of peptides was obtained.

Secondary Structure Predictions and Transmembrane Arrangement of Mammalian Porin. Further investigation of the topology of the membrane-bound porin was carried out

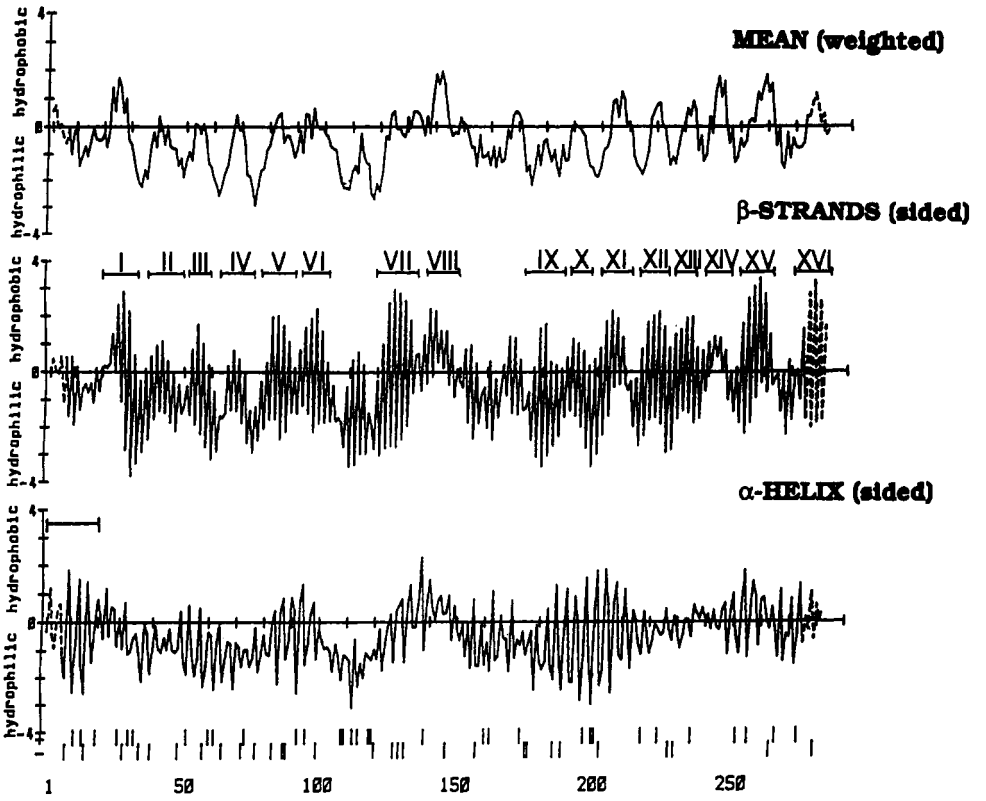

FIGURE 8: Sided-hydropathy profiles of porin from human B-lymphocytes. The sided-hydropathy values for sided α-helices and β-sheets and the corresponding averaged hydropathy values were obtained by summing up the hydropathy indices of the respective residues (Kyte & Doolittle, 1982), using the following weights as given in parentheses. (A) Weighted average: residues n (1.0), $n \pm 1$ (0.5), $n \pm 2$ (0.5), and $n \pm 3$ (0.5). (B) Sided β-sheets: residues n (1.0), $n \pm 2$ (1.0), and $n \pm 4$ (0.5). (C) Sided α-helix: residues n (1.0), $n \pm 3$ (0.5) (distance 1 turn, 40°), $n \pm 4$ (0.5) (1 turn, 60°), and $n \pm 7$ (0.5) (2 turns, 20°). Thus three values were obtained for each residue n and plotted above the number of the residue. The comparison of the three traces allows the prediction of different structural domains. In the weighted average plot, hydrophobic domains are detected, while amphipathic segments appear to be neither hydrophobic nor hydrophilic. Possible amphipathic α-helices show an alteration between hydrophobic and hydrophilic residues every third or fourth residue in the sided α-helical plot, i.e., at the frequency with which an α-helix turns around its central axis (3.6 residues per turn). Possible sided β-sheets can be detected in the sided β-sheet profile by an alternation between hydrophobic and hydrophilic values every second residue. Tentative predictions of possible amphipathic membrane-spanning β-strands is indicated by bars (I–XVI). Charged residues are indicated at the bottom of the figure (up, positive; down, negative).

by means of a more refined analysis of the human porin sequence and by looking for amphipathic secondary structures spanning the membrane. The first 18 amino acids were suggested to form an amphipathic α-helix, as already shown in Kayser et al. (1989). The remaining sequence should mainly contain sided β-strands. A similar pattern has been indicated for the *S. cerevisiae* and *N. crassa* porin, where 15 stretches of sided β-sheets were predicted (Kleene et al., 1987). In Figure 8 the results of our computer-based predictions are shown. We looked for sided-hydropathy profiles in possible α- and β-structures. The analysis was performed with amino acid strings of different lengths and giving different weights to the residues (see legend to Figure 8). We found a number of 16 transmembrane β-strands to be the most likely pattern. Some uncertainty existed only in defining the segment 188–198 as a possible transmembrane β-strand segment. In Figure 9 the transmembrane arrangement of human mitochondrial porin based on secondary structure predictions is shown. Most of the protein should be embedded in the phospholipid bilayer. The amphipathic N-terminal α-helix and two major loops (102–123 and 148–173) should protrude into the external water phase. The most important antigenic epitopes should be localized in these regions, since the C-terminal third of the protein seems poorly detectable by polyclonal antisera, as demonstrated by the lack of reactivity of the C-terminal peptides generated by protease V8 and chymotrypsin (Figures 5A and 6A, respectively). We found 29% and 24% homology, respectively, between human and *N. crassa* or *S. cerevisiae* porin (Kayser et al., 1989). We analyzed the distribution of sequence homologies between human and fungal porins to verify if homologies were concentrated in special regions of the protein. Among the membrane-spanning segments, the starting α-helix (amino acids 1–22) showed 8 identities and 3 conservative exchanges between human and *N. crassa*, or human and yeast, porin. This would confirm an important role of this helix. The β-strands numbered III (50–61 in the human sequence), VI (91–103), X (186–197), and XII (218–227) had about 50% homology between human and *N. crassa* porin. In the C-terminal region more homologies were observed between human and yeast than between human and *N. crassa*, with the XVI strand (273–282), containing the C-terminus, 60% identical in the two organisms. In the water-exposed regions of the protein, loop II (148–173) showed 9 identical and 4 conservative exchanges between human and *N. crassa* out of 26 total residues. The segment connecting β-strands XIV and XV (249–256) had 5 (human/*N. crassa*) and 6 (human/yeast) homologous residues out of 8 amino acids. No special homology could be observed in the remaining part of the sequence. By site-directed mutagenesis of yeast porin, Blachly-Dyson et al. (1990) identified 14 functionally important sites, distributed throughout the length of the molecule. Mutation at one or more of those 14 sites altered the selectivity of the channel for ions. Comparison with our model showed that the 14 essential 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

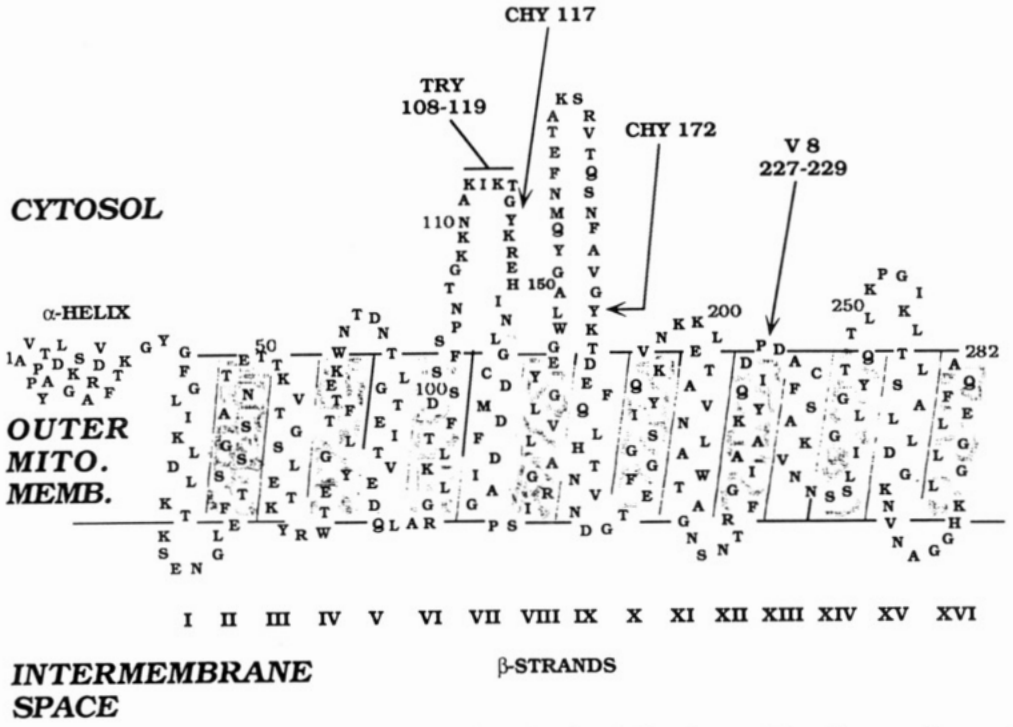

FIGURE 9: Model of transmembrane arrangement of the polypeptide chain of porin from human B-lymphocytes. The starting amphipathic α-helix lies on the external surface of the membrane. Sixteen amphipathic β-strands cross the membrane. The loops containing the cleavage sites of protease V8, trypsin, and chymotrypsin, indicated by arrows, are exposed to the cytosol.

properties seem to be identical. The conserved "essential" residues are D_{15}, K_{19}, D_{29}, K_{60}, and K_{95}.

In contrast with data obtained for *N. crassa* porin (Kleene et al., 1987), human porin did not show any homology with the members of the mitochondrial metabolite carrier family; neither did it have the tripartite structure typical of these proteins (Runswick et al., 1990).

Discussion

In this paper we have tried to assign a transmembrane arrangement to the mitochondrial porin, the protein structure that forms the wide, unspecific channel of the mitochondrial outer membrane. Our model of arrangement is based on the following experimental results which are discussed in detail below: (i) the N-terminal region of porin is an amphipathic α-helix exposed to the water phase; (ii) the C-terminus is not exposed to the water phase; (iii) 16 amphipathic β-strands which can cross the membrane have been predicted by computer analysis; (iv) four specific cleavages have been detected and the putative sites of cleavage assigned; (v) no difference was found between intact and broken mitochondria, neither in ELISA nor in proteolytic experiments.

(i) The water-exposed localization of the N-terminus of porin has been demonstrated by ELISA experiments that used antisera raised against synthetic peptides with the human porin N-terminus sequence. The reaction of these antisera with porin in its native location in the mitochondrial outer membrane was detected by direct reaction of mitochondria with antibodies and by back-titration. The N-terminal region of porin should form 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* (Kayser et al., 1989). Moreover, the antibodies against the human N-terminus were found to be active against porin from *D. melanogaster* (De Pinto et al., 1989), confirming the existence of a relevant structural similarity in the N-terminal region. It should be recalled that it has been claimed that the N-terminal α-helix is involved in the import pathway of the protein, which has no cleavable presequence (Kleene et al., 1987).

(ii) The lack of porin cleavage in membranes of intact or broken mitochondria by carboxypeptidase A has been interpreted by us to indicate that the C-terminus is not exposed to the water phase. Our hypothesis is that the C-terminal end of porin is embedded in the membrane, where is should form the sixteenth amphipathic β-strand. This contention is corroborated by the computer-based secondary structure analysis made in this paper (Figure 8) and by previously published predictions for the yeast porin (Forte et al., 1987; Blachly-Dyson et al., 1989). Also 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. This finding would explain why the antiserum against the whole porin failed to detect the C-terminal fragments generated upon proteolysis with protease V8 and chymotrypsin (Figures 5 and 6). 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). From the model shown here (Figure 9) and by Blachly-Dyson et al. (1990) for yeast porin, one can assume that the C-terminus plays an important role in anchoring the protein to the membrane and, thus, in determining the topological arrangement of the porin molecule.

(iii) Computer-based search for secondary structures in the human porin predicted the presence of an amphipathic α-helix (discussed above) and 16 sided β-strands. There would not be any hydrophobic regions long enough to span the bilayer as hydrophobic α-helices. On the other hand, the sided β-strand is a structure seldom found in membrane protein al-

though it is typical of porins both from bacteria (Weiss et al., 1991) and from mitochondria (Kleene et al., 1987). It 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 (49.3% of polar residues). The secondary structure prediction of the porin molecule is more difficult than that of an α-helix-type membrane protein since the transmembrane segments are rather short and therefore difficult to distinguish. We found a number of 16 transmembrane β-strands to be the most probable arrangement. We have some uncertainty only in defining the segment 188–198 as a possible transmembrane β-strand segment. On the other hand, a model with 15 β-strands rather than 16 would be in contrast with our experimental results, as will be discussed below. In the literature we found that 12 (Blachly-Dyson et al., 1989, 1990) or 19 (Forte et al., 1987) β-strands were predicted from the analysis of the yeast porin sequence. The 12-strand model of Blachly-Dyson et al. (1990) omits large parts of the sequence from the membrane since mutations in that range did not alter the channel behavior of their mutants. We consider it very dangerous to deduce the topology from functional data only, since not all of the residues in the membrane have to be involved in forming the channel. Thus, only 5 residues considered functionally essential by Blachly-Dyson et al. (1990) are conserved in human porin. On the other hand, Forte et al. (1987) have published a 19 β-strand model, based on the Delphi program. It is not yet clear if the channel of the mitochondrial outer membrane is made up of a single polypeptide or of two or even more subunits. By postulating 15–19 transmembrane β-segments, a single porin chain can be folded into a "β-barrel"-type structure which has pore dimensions consistent with those determined by the biophysical analysis (Colombini, 1979; Benz, 1985; De Pinto et al., 1987a). Interestingly, Weiss et al. (1991), who determined the crystal structure of porin from *Rhodobacter capsulatus* at 1.8-Å resolution, found 16 β-strands crossing the membrane to form a "β-barrel" structure which was large enough to form a channel. Also the most recent results from electron microscopy of frozen-hydrated *N. crassa* outer mitochondrial membrane crystals favor a monomer channel. Mannella et al. (1989) showed that a β-barrel consisting of a 3.8-nm-diameter α-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 that 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; Benz, 1985; De Pinto et al., 1987a). The only report that claimed the porin channel to be a dimer was based on the hydrodynamic parameters of the purified porin in Triton X-100 micelle (Linden & Gellerfors, 1983).

(iv and v) We have subjected mitochondria to the action of several proteases, looking for cleavage sites located in water-exposed domains of porin. It should be pointed out that, although the proteolysis was performed at 37 °C, only a few of the proteases assayed were able to cleave porin and that the cleavage occurs in a very specific way, usually at only one site. This result is in agreement with the fact that porin is deeply embedded in the phospholipid bilayer (Mihara et al., 1982; Freitag et al., 1982b). On the basis of the number of the fragments generated upon enzymatic digestion of porin and their molecular weight, we propose that the cleavage site of trypsin is located between residues 108 and 119 (where 5 lysines and 1 arginine are present), that of chymotrypsin at positions 117 and 172, and that of protease V8 at D_{227} or D_{229}. The latter conclusion is also supported by the observation that porin was cleaved at pH 8 and not at pH 4, i.e., at an aspartic acid residue.

In our model all large hydrophilic domains and all the cleavage sites are exposed to the outside of the membrane. This model explains why we got the same results with ELISA and enzymatic digestion in intact and broken mitochondria. Porin is completely cleaved by protease V8, trypsin, and chymotrypsin in intact mitochondria. This excludes an antiparallel orientation of porin molecules in the outer mitochondrial membrane, since in this case only part of the molecules would have been cleaved in intact mitochondria. The experiments of immunotitration show that the amounts of antibodies bound to intact or broken mitochondria are the same, suggesting that virtually all the epitopes are exposed to the outside. Again, in the case of an antiparallel orientation of porin molecules, we would have found more binding to membranes as compared to mitochondria.

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.

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Fluorescence Study of a Mutant Cytochrome b_5 with a Single Tryptophan in the Membrane-Binding Domain†

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ABSTRACT: Fluorescence studies of cytochrome b_5 are complicated by the presence of three tryptophans, at positions 108, 109, and 112, in the membrane-binding domain. The cDNA for rabbit liver cytochrome b_5, isolated from a λgt11 library, was used to generate a mutated mRNA where the codons for tryptophans-108 and -112 were replaced by codons for leucine. The sequence was expressed in *Escherichia coli* and the mutant protein was isolated. This mutant protein had the expected absorption spectrum, and its amino acid composition was confirmed by amino acid analysis and by DNA sequencing of the construct. The fluorescence emission spectrum of the mutant is blue-shifted and is narrower than that of the native protein. The quantum yield of the mutant protein, per molecule, is only 60% of that of the native protein, and the enhancement when bound to lipid vesicles or detergent micelles is higher for the mutant. Fluorescence anisotropy measurements and quenching studies using brominated lipids suggest that the fluorescence of the native protein is due to tryptophans-109 and -108 while tryptophan-112 does not emit but undergoes nonradiative energy transfer to tryptophan-108. With this mutant, it was shown that incomplete energy transfer from tyrosines-126 and -129 to tryptophan-109 occurs when the membrane binding domain is inserted into lipid vesicles, which suggests that the membrane-binding domain does not exist in a tight hairpin loop.

Cytochrome b_5 (b_5)[1] is an integral membrane protein found in the endoplasmic reticulum of liver cells and other cells. It plays a central role in metabolism by virtue of its involvement in fatty acid desaturation (Holloway, 1983) and elongation (Nagai et al., 1983), cholesterol metabolism (Grinstead & Gaylor, 1982), and the metabolism of xenobiotics (Tamburini & Schenkman, 1988). In addition, because of its relative ease of isolation, it has become a popular model for studies of lipid–protein interactions and for evaluating various physical techniques for the study of membrane proteins in general. It should be noted, however, that it is probably only representative of a subset of membrane proteins. For many of these aforementioned studies, use has been made of the fluorescent

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[1] Abbreviations: b_5, cytochrome b_5 (the complete 133 amino acid residue protein); rabbit b_5, native cytochrome b_5 isolated from rabbit liver; mutant b_5 cytochrome isolated from *E. coli* with Trp-108 and Trp-112 of the native sequence both replaced by Leu; NPP, membrane-binding domain of cytochrome b_5 which is released from cytochrome b_5 by trypsin treatment; BRPC, 1-palmitoyl-2-(dibromostearoyl)phosphatidylcholine; Tris buffer, 5 mM Tris–acetate containing 1 mM EDTA (pH 8.2); OG, octyl β-D-glucoside; TX100H, Triton X-100 hydrogenated; Γ, full width at half-maximal height of a spectral band.

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