Structure and Orientation of Two Voltage-dependent Anion-selective Channel Isoforms

Two VDAC (voltage-dependent anion-selective channel) isoforms were purified from seed cotyledons of Phaseolus vulgaris by chromatofocusing chromatography. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy was used to study the structural properties of the two isoforms reconstituted in a mixture of asolectin and 5% stigmasterol. The IR spectra of the two VDAC isoforms were highly similar indicating 50 to 53% anti-parallel β-sheet. The orientation of the β-strands relative to the barrel axis was calculated from the experimentally obtained dichroic ratios of the amide I β-sheet component and the amide II band. Comparing the IR spectra of the reconstituted VDAC isoforms with the IR spectra of the bacterial porin OmpF, for which a high resolution structure is available, provided evidence for a general structural organization of the VDAC isoforms similar to that of bacterial porins. Hydrogen-deuterium exchange measurements indicated that the exchange of the amide protons occurs to a higher extent in the two VDAC isoforms than in the OmpF porin.

Mitochondria are surrounded by two distinct membranes. The mitochondrial outer membrane is freely permeable for small hydrophilic solutes up to approximately 6 kDa. This property has been attributed to the presence of a pore forming protein, the voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (1). Biophysical properties of VDAC channels inserted in planar lipid bilayers have been studied in a large variety of organisms. VDAC channels switch from a high conducting fully open state (3.6 to 4.5 nS in 1 M KCl) at low voltages to several low-conducting substates upon application of voltages higher than ±20 mV. VDAC is slightly anion selective in the fully open state but switches to cation selectivity in the low-conducting substates (2). In these substates, the flow of negatively charged metabolites such as succinate, citrate, phosphate (3), and adenine nucleotides (4, 5) through the channel is strongly reduced compared with the fully open state. Therefore, VDAC is thought to regulate the mitochondrial metabolism by regulating the flux of metabolites across the mitochondrial outer membrane.

VDAC channels are involved in different cellular events like the induced release of cytochrome c, which constitutes an early step in apoptosis (6). VDAC is the binding site for cytoplasmic enzymes like glycerol kinase and hexokinase (7 and (7) for the cytoskeleton (8). The different VDAC isoforms that exist in yeast, plants, and mammals could be involved in specific functions. Two human VDAC isoforms have different binding affinities for hexokinase (9). Plant VDAC isoforms have slightly different electrophysiological properties (10), and mitochondria isolated from yeast vDAC minus mutants show differences in their outer membrane permeability to NADH depending on which of the three mouse VDAC isoforms was expressed (11). Therefore, it seems reasonable to assume that the multitude of functional properties that are attributed to VDAC could arise from the presence of different VDAC isoforms in mitochondria.

VDAC proteins from different far related organisms generally exhibit poor sequence homologies (~30%) but VDAC from plants seem to have more conserved amino acid sequences (12). Despite poor sequence homologies, it is assumed that all VDACs have a rather conserved β-barrel structure similar to that of bacterial porins. This assumption arose from secondary structure predictions (13–15) that suggested anti-parallel β-strands as the main structural feature. CD measurements of purified yeast VDACs (16, 17) indicated a high β-sheet content. Electron microscopic studies of two-dimensional crystals of fungal VDAC and low-resolution images of human VDAC crystals suggested that VDAC proteins form a pore (18, 19). Although the molecular structure of several bacterial porins has been resolved at the atomic level (20, 21), no high-resolution VDAC crystals are available.

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EXPERIMENTAL PROCEDURES

Materials—Seeds from P. vulgaris var. “Streamline” were purchased from a local store. Percoll, chromatofocusing media PBE 94, and Polybuffer PB 96 were from Amersham Pharmacia Biotech. Asolectin (soybean phosphatidylcholine) and stigmasterol were obtained from Sigma. Genapol-X-080 was purchased from Fluka and D2O from Merck. Bio-Beads were from Bio-Rad. The bacterial porin, OmpF from E. coli was a kind gift from Dr. T. Schirmer (University of Basel, Basel, Switzerland).
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VDAC Purification—The two VDAC isoforms were purified from seeds of P. vulgaris var. “Streamline” following the procedure described previously with minor modifications. The two VDAC proteins were purified and separated in a single chromatofocusing chromatography step, i.e., the HTP batch described in the previous protocol (22) was omitted. Briefly, seeds from P. vulgaris were soaked in tap water for 24 h and mitochondria were isolated from the cotyledons by differential centrifugation steps and further purified on a 28% Percoll gradient (23). Then, the final mitochondrial pellet (300–400 mg of protein, 20–25 mg/ml) was mixed with an equal volume of 2-fold concentrated chromatofocusing start buffer (see below) supplemented with 4% (v/v) Genapol-X-080 to stabilize the membranous proteins. This suspension was aliquoted (2 ml) and frozen at ~–20 °C until use. A 10 ml (1.6 × 5 cm) chromatofocusing column was prepared with polybuffer exchanger media (PBE 94, Amersham Pharmacia Biotech) and equilibrated with the start buffer containing 25 mM ethanolamine, 4 mM urea, 0.1% (v/v) Genapol-X-080, pH 7.9. A 2 ml mitochondria suspension in start buffer (containing 2% (v/v) Genapol-X-080) was thawed at room temperature and incubated for 30 min at 4 °C. Insoluble material was removed by centrifugation at 10,000 × g for 5 min (Beckman, Allegra 21R). The supernatant, containing approximately 20 mg of protein, was loaded onto the chromatofocusing column and proteins were eluted with 10% (v/v) Polybuffer 96 (PB 96, Amersham Pharmacia Biotech), 4 mM urea, 0.1% (v/v) Genapol-X-080 at pH 7.0. The fractions containing the individual VDAC isoforms from 3 chromatofocusing columns (about 25 ml) were pooled and concentrated about 20-fold (Centricon-30, Amicon). The elution buffer was exchanged against 1 ml Heps, 0.1% (v/v) Genapol-X-080, pH 7.2, by gel filtration (Sephadex G-75, Amersham Pharmacia Biotech) at a flow rate of 7.5 cm/h. The proteins eluted at a concentration of about 0.07 to 0.12 mg/ml.

Reconstitution of the Purified VDAC Isoforms—850 μg of purified aselenin (24) and 50 μg of stigmasteryl were dissolved in chloroform and the solvent was evaporated under a stream of N2 to have a thin lipid film which was dried further overnight under vacuum. The dry lipids were resuspended in 2 ml of protein solution (~0.1 mg of protein/ml). The lipid/protein/detergent mixture was incubated 30 min at room temperature under constant agitation followed by 3 freeze/thawing cycles. The removal of detergent was achieved by 4 subsequent additions of 40 mg of Bio-Beads (previously washed with methanol and water). Incubation times were 45, 45, 30, and 30 min under constant agitation at 4 °C. After removal of the final Bio-Beads, the proteoliposome suspension was diluted with 1 ml Heps, pH 7.2, to a total volume of 4.5 ml. The proteins associated with the lipid vesicles were pelleted at 37,000 rpm for 2 h (Beckman L7, SW-60 rotor). This washing step was repeated twice. Finally, the proteoliposomes were suspended in 20–30 μl of 1 ml Heps, pH 7.2. The proteoliposomes had a lipid to protein ratio of about 10.1 (w/w). Protein recovery was in the range of 25–35%.

Bacterial porin was diluted in 2 ml of 1 M Heps, 0.1% (v/v) Genapol, pH 7.2, to a similar concentration as were the VDAC proteins and reconstitution was then carried out as above described for VDAC. Liposome blanks were prepared with 2 ml of buffer in the absence of protein.

In order to demonstrate the association of VDAC proteins with the lipids, the proteoliposomes were centrifuged on a 32 to 5% (w/v) sucrose gradient with a 40% (w/v) sucrose cushion for 16 h at 35,000 rpm (Beckman L7, SW-60 rotor). The gradient was then fractionated from the bottom of the tube. Turbidity of each fraction was measured at 405 nm and protein was detected with the BCA assay (Pierce).

IR Spectroscopy—Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were obtained on a PerkinElmer 1720 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector as described elsewhere (25). The spectra were recorded at a nominal resolution of 4 cm−1 and 128 scans were averaged for each measurement. The internal reflection element was a germanium ATR plate (50 × 20 × 2 mm, Harrick EJ 2121) with an aperture angle of 45°, yielding 25 internal reflections. The spectrophotometer was continuously purged with dry air. Spectra with polarized incident light and kinetics of hydrogen/deuteration exchange were measured on a Bruker IFS-55 spectrophotometer equipped with liquid nitrogen-cooled mercury cadmium telluride detector and a polarizer mount assembly (KRS-5 polarizer).

Sample Preparation—20–30 μl of reconstituted protein were spread on one side of the ATR plate. The solvent was slowly evaporated under a continuous stream of nitrogen. So, 15–30 μg of protein were deposited on the ATR plate.

Analysis of the Secondary Structure—The determination of protein secondary structure was based on the multivariate statistical analysis method for band shape recognition similar as previously reported (26). This analysis used a commercially available partial least-square package to determine fractional secondary structure composition (PLSPlus 2.1 from Spectra Calc, Galactic Industries, Salem NH). A reference set (RasSp50) was generated by collecting ATR-FTIR spectra of 50 proteins with known x-ray structures selected to represent a wide range of α-helix and β-sheet compositions as well as 60 different protein domain folds. Before analysis, all spectra were normalized so that the PLS algorithm was forced to use band shapes of the amide I and amide II bands (between 1720 and 1500 cm−1) rather than absolute intensities. Cross-validation procedures using the RasSp50 protein spectra have shown RMS structure determination errors of ±4.5% for α-helix and ±6.3% for β-sheet.

Orientation of the Secondary Structure—ATR-FTIR spectroscopy provides information on the orientation of the peptide secondary structure embedded in the hydrated lipid film where the lipid acyl chains are oriented perpendicular to the surface of the ATR plate (27). The protein spectra were recorded with parallel and perpendicular polarized light with respect to the plane of incidence, averaging 512 scans at a resolution of 2 cm−1. To determine the dichroic ratio of the different secondary structure components, the amide I band (1700 to 1600 cm−1) was decomposed by a least-square curve-fitting procedure using a Cauchy (Lorenzian/Gaussian) function as described previously (25). The integrated areas corresponding to the β-sheet components (near 1630 cm−1) were determined, and the ratio of the integrated areas from the two polarized spectra then yielded the dichroic ratio of the β-sheet, RβATR. The procedure was repeated to determine the dichroic ratio of the α-helix component (at ~1655 cm−1). The dichroic ratio of the amide II band was determined from the ratio of the integrated areas between 1590 and 1505 cm−1 from the two polarized spectra. The dichroic ratio of the lipid ν(C=O), called below Rν(C=O), was obtained from the parallel and perpendicular polarized spectra. Rν(C=O) was used to compute the film thickness and the values of the electric field components Eα, Eβ, and Eν at 1631 cm−1 as described previously (28). For these calculations refractive indexes of 4.0 and 1.44 were used for the Genapol and the sample film, respectively.

In a β-sheet the amide I transition dipole moment is oriented parallel to the C=O bond which is oriented perpendicular to the β-strand axis. The N-H bending contributes mainly to the amide II transition, because two symmetry axes are needed to describe the sheet orientation, the combination of the dichroic ratios from both amide I and amide II bands are required to determine the orientation of the anti-parallel β-strands. In a barrel structure, axial symmetry around the barrel symmetry axis can be partially obtained (29–31). The orientation of the β-strands was calculated according to Marsh (30, 31).

Kinetics of the Hydrogen/Deuterium Exchange—Deuteration kinetics were carried out as described previously (32, 33). A computer program controlled the kinetic measurements. Prior to each experiment 10 spectra were recorded to verify the stability and the reproducibility of the system. At time 0 the sample was flushed with D2O-saturated N2 at a flow rate of 4 liters/min. For each spectrum 24 scans were accumulated at a resolution of 2 cm−1. Background and water vapor spectra were subtracted from the kinetic spectra as reported (32).

The areas of the amide I and amide II bands were calculated by integration of the spectra between 1700 and 1600 cm−1 and 1590 and 1505 cm−1, respectively. The area of the amide II band decreases as deuteration of the protein proceeds. To take into account variations of the overall spectral intensities related to film swelling upon hydration in the first minutes of the measurement, the amide II/amide I ratio was determined for each spectrum. Thus, the H/D exchange was monitored as the evolution of the ratio of amide II/amide I areas, expressed as percent of non-exchanged amide protons. The amide II/amide I ratios of the fully exchanged spectra were taken as 100%. The value of the 0% value corresponds to a zero absorption in the amide II region (34).

We observed in our experiments a considerable absorbance of the lipids in the amide I and amide II region that overlapped the protein spectra. To obtain the pure protein spectra, the spectra of H/D exchange kinetic of a liposome blank were recorded under identical conditions. A computer program subtracted the spectra of a liposome blank from each corresponding sample spectrum. To account for varying absorbance intensities in sample and lipid spectra, a subtraction coefficient was used to cancel the lipid ν(C=O) area integrated between 1705 and 1770 cm−1.

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RESULTS

VDAC Purification—As a modification to the previously reported protocol (22), purification of the two VDAC isoforms was achieved in a single purification step. Mitochondria were isolated from the cotyledons of 24-h imbibed kidney beans (P. vulgaris) seeds and solubilized with 2% (v/v) Genapol X-080 and 4 M urea. The solubilized proteins were loaded on and eluted from a chromatofocusing column using a pH gradient from pH 9.4 to 7.0. Fig. 1 shows a Coomassie Blue-stained SDS gel with the two individual VDAC proteins which were separated and purified by chromatofocusing chromatography (Fig. 1, lanes 1 and 3). The two isoforms are called VDAC 31 and VDAC 32 according to their respective apparent molecular mass. SDS gels loaded with high amounts of protein (10–20 μg) confirm the high purity of the protein preparation (Fig. 1, lanes 2 and 4). The staining intensities reflect the abundance of each isoform in the seed mitochondria (22).

Reconstitution—The purified VDAC isoforms were reconstituted in asolectin supplemented with 5% (w/w) stigmasterol. The detergent was removed from the mixed lipid-detergent-protein micelles by adsorption on Bio-Beads to promote the formation of proteoliposomes. Formation of proteoliposomes was demonstrated by the co-migration of lipids and proteins during a sucrose gradient centrifugation (Fig. 2). No protein aggregates were detected at the bottom of the tube. Fig. 2 shows the distribution pattern for the VDAC 31, VDAC 32, and material porin OmpF for which a high-resolution crystal structure is available (Fig. 3A). Fourier deconvolution of the spectra allows a more detailed analysis of the spectral composition (Fig. 3B). Spectra of VDAC 31 and VDAC 32 are almost identical in the region of the amide I band between 1700 and 1600 cm⁻¹. The amide I bands are characterized by a major component located around 1631 cm⁻¹ which can be assigned to β-sheet. The position of the major component at 1631 cm⁻¹ and a weak component at about 1694 cm⁻¹ in the amide I region, together with the major amide II band at 1535 cm⁻¹, indicate that the β-strands are in an anti-parallel configuration (35). The shape of the amide I bands of the two VDAC isoforms are very similar to that of the OmpF porin, suggesting a similar secondary structure composition. The IR spectrum of the OmpF is virtually identical to that previously reported (36, 37). The secondary structure content of the VDAC isoforms resembles that of the OmpF porin, suggesting a similar secondary structure composition. The IR spectrum of the OmpF is virtually identical to that previously reported (36, 37). The secondary structure content of the VDAC isoforms and of the OmpF is reported in Table I. The secondary structure of all three proteins is very similar. It must be noted here that the variations observed are in the range of the error made in evaluating the secondary structure. The secondary structure determined here for OmpF closely matches the crystal structure (Table I) (21).

Orientation of the Secondary Structure—The ATR-FTIR spectroscopy technique provides information about the orientation of proteins inserted in a lipid bilayer. Protein spectra were recorded with parallel and perpendicular polarized incident light (Fig. 4A). The dichroic spectra (i.e. parallel minus perpendicular polarized spectrum) of the two VDAC isoforms and of the OmpF are illustrated in Fig. 4B. The three spectra...
show a positive dichroism signal for the main component of the amide I band at $-1630$ cm$^{-1}$ and a positive deviation of the amide II band indicating that the $\beta$-sheet structure has a preferred orientation. A similar dichroic spectrum of porin has been reported previously (37). To determine the orientation of the $\beta$-strands, the amide I band was decomposed into its different components by a least-square curve-fitting procedure to obtain the dichroic ratio $R_{\text{ATR}}$ of the $\beta$-sheet component. For a quantitative determination of the $\beta$-strand orientation, the dichroic ratios of both the amide I band (here $R_{\text{ATR}}$) and the amide II band have to be determined because the transition dipole moment of $\beta$-strands does not exhibit uni-axial symmetry (29). The orientation $\Theta _{\parallel }$ of the amide I transition dipole moment with respect to normal to the membrane is related to the strand tilt, $\beta$, by $\Theta _{\parallel } = (\pi/2) - \beta$. For the amide II transition dipole moment, which is oriented parallel to the strand axis, $\Theta _{\parallel } = \beta$. For a regular $\beta$-barrel, the transition dipole moments are distributed with axial symmetry around the barrel axis and the orientation of the transition dipole moments is related to the dichroic ratios $R_{\text{ATR}}$ by,

$$
\frac{P_{\text{R}}(\cos \Theta)}{P_{\text{R}}(\cos \alpha)} = \frac{E_x^2 - E_y^2 \times R_{\text{ATR}} + E_z^2}{E_x^2 - E_y^2 \times R_{\text{ATR}} - 2E_z^2},
$$

(Eq. 1)

where $P_{\text{R}}(x) = (1/2)(3x^2 - 1)$ is a second order Legendre polynomial and $\alpha$ is the orientation of the barrel axis relative to the membrane normal (30, 31). This equation can be written for either the amide I band or the amide II band, so that two equations are obtained to calculate the order parameters. $E_x$, $E_y$, and $E_z$ are the amplitudes of the electric field in a coordinate system, where $x$ is perpendicular to the ATR plate surface, $y$ and $z$ are in the ATR-plate plane with $z$ in the incidence plane and $y$ perpendicular to the incidence plane. The values of $E_x$, $E_y$, and $E_z$ were computed considering the real film thickness (see "Experimental Procedures"). The tilt angle, $\beta$, was calculated from the experimental dichroic ratios of both the amide I ($R_{\text{ATR}}$) and the amide II bands using Equation 1. The $\beta$-strands

![An ATR-FTIR Spectroscopy Study of Two VDAC Isoforms](image)
in all three proteins are tilted at a very similar angle of -45° to 47° relative to the barrel axis (Table II). A value of 45° for the strand tilt in OmpF is in good agreement with the mean strand orientation deduced from the crystal structure (21, 31). The order parameter corresponding to the α angle is similar for the two VDAC isoforms (\(P_\alpha(cos \alpha) = 0.36\) and 0.45 for VDAC 31 and VDAC 32, respectively) but is significantly smaller than for OmpF (\(P_\beta(cos \beta) = 0.66\)). This point will be discussed later. The value for OmpF agrees well with data reported by Marsh (31). The contribution of the α-helical component of the amide I band was too low (-10%) to have enough accuracy to calculate the orientation of the α-helix.

**Kinetics of the Hydrogen/Deuterium Exchange**—The kinetic

**TABLE II**

Orientation of the β-strands in VDAC and OmpF

| Protein  | \(R^\text{STR}_\beta\)  | \(R^\text{STR}_{\text{amide II}}\) | \(\beta\) |
|----------|-------------------------|---------------------------------|---------|
| VDAC 31  | 1.84 (± 0.04)           | 1.76 (± 0.05)                   | 47 (± 1.2) |
| VDAC 32  | 1.94 (± 0.07)           | 1.89 (± 0.05)                   | 46 (± 0.3) |
| OmpF     | 1.73 (± 0.12)           | 1.72 (± 0.14)                   | 45 (± 0.25) |

of H/D exchange of the amide protons is monitored by evaluating the amide II/amide I area ratio. As amide II arises predominantly from the peptide N-H bending, the rate of H/D exchange is related to both the solvent accessibility of the NH amide groups of the peptide bond (which is related to the tertiary structure) and to the stability of secondary structure elements. The kinetics of H/D exchange was followed by monitoring the decrease of the area of the amide II band as a function of time to D₂O exposure. A series of ATR-FTIR spectra recorded upon H/D exchange of reconstituted VDAC 31 appears in Fig. 5. Clearly, the amide II area (near 1530 cm⁻¹) decreases as deuteration proceeds due to a shift of the amide II band upon deuteration to near 1445 cm⁻¹ (called amide II'). The deuteration kinetics of the two VDAC isoforms and of the OmpF porin are compared in Fig. 6. The H/D exchange occurs to different extents in VDAC and OmpF. For both VDAC isoform more than 60% of the amide hydrogens are exchanged after 10 h. On the contrary, under the same experimental conditions, only about 35% of the OmpF amide protons are exchanged which is consistent with previously reported values (36). Although the decrease of the amide II intensity measures the global H/D exchange, the exchange kinetics can quantitatively be analyzed as follows. The exchange curves follow a multiexponential decay. The H/D exchange is a first-order reaction involving different groups, i, of amide protons characterized by a common period \(T_i\).
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**FIG. 6.** Deuterium/hydrogen exchange kinetics of VDAC 31 (squares), VDAC 32 (circles), and OmpF (triangles) reported as percent non-exchanged hydrogen. Deuteration percentage was evaluated from the evolution of the area amide II/area amide I ratio upon exposure to D$_2$O. The vertical bars represent the standard deviation of the mean of two (VDAC 31) or three (VDAC 32) independent experiments.

\[
H(t) = \sum a_i \exp(-t/T_i)
\]  
(Eq. 2)

where $a_i$ is the number of amino acids of the group $i$. Within the experimental reproducibility limits, a nonlinear fitting of the experimental curves (Fig. 6) could be performed with three exponentials indicating that the exchange can be described as the exchange of three groups of amide protons with the proportions $a_1$, $a_2$, and $a_3$ characterized respectively by the periods $T_1$, $T_2$, and $T_3$. Each time constant ($T_1$, $T_2$, and $T_3$) was very similar for the two VDAC isoforms. To compare the proportions of each group of amide protons, the $T_i$ values of the two VDAC kinetics were replaced by their mean values ($T_1 = 1.1$, $T_2 = 10$, and $T_3 = 2300$ min) and a second fitting was performed using $T_1$ constrained to their mean values (32). The number of amide protons (in % of total amide protons) in each group are reported in Table III. The three groups of the OmpF amide protons had time constants of $T_1 = 1.3$, $T_2 = 28$, and $T_3 = 5637$ min (Table III). Only 17% of the OmpF amide protons but 30% of the VDAC amide protons exhibit a fast exchange rate with comparable time constants of $T_1 = 1.3$ and 1.1 min, respectively. On the contrary, two-thirds of the OmpF amide protons exhibit a very slow exchange rate ($T_3 = 5637$ min). In comparison, only 48% of the VDAC amide protons exhibit a slow exchange rate but still faster than in OmpF (Table III).

**DISCUSSION**

Two VDAC isoforms were purified from kidney bean cotyledons in a single purification step using chromatofocusing chromatography as previously reported (22). Highly purified proteins were reconstituted in a mixture of asolectin and stigmasterol, a major sterol of plant membranes. Sterols have been found to be associated with VDAC isolated from Neurospora crassa and bovine heart mitochondria (38, 39). It has been suggested recently that sterols are essential for the proper folding of VDAC (40).

The IR spectra of the reconstituted VDAC isoforms are strikingly similar displaying characteristics typical for an anti-parallel $\beta$-pleated sheet. The two spectra could indeed almost be superimposed, strongly suggesting that the two isoforms have an almost identical secondary structure. Quantitative analysis indicated that about 50 to 53% of the amino acid residues are in $\beta$-sheet conformation. Moreover, the percentages of the different secondary structure elements calculated from the amide I and amide II bands associated to OmpF and VDAC were almost identical. The agreement of the secondary structure of OmpF determined by IR analysis and from the crystal structure (Table I) strongly supports the validity of the structures proposed for VDAC.

Despite the remarkable similarity between the VDAC and the OmpF IR spectra a few differences can be observed. The main peak of the VDACs amide I bands (1631 cm$^{-1}$) is slightly shifted toward a higher wavenumber compared with the peak of the OmpF spectrum (1629 cm$^{-1}$). Kleefel et al. (36) correlated the position of the main $\beta$-sheet peak with the length (i.e. the number of amino acids) of the $\beta$-strands. It has been found that the peak at around 1630 cm$^{-1}$ shifted toward higher wavenumbers for shorter $\beta$-strands. The slight shift in the VDAC spectra could therefore be an indication for shorter $\beta$-strands in VDAC than in OmpF (~12 residues on the average). The $\alpha$-helix content in VDAC (~9 to 13%) seems slightly higher than in OmpF, visible as a shoulder in the VDAC IR spectra at about 1660 cm$^{-1}$ (Fig. 3). This observation could correlate with the assumption that the N terminus of VDAC forms an amphipathic $\alpha$-helix. It has been suggested that this $\alpha$-helical N terminus might play a particular role in protein import into the mitochondrial outer membrane and in the voltage-dependent gating of the channel (41–43).

The possibility to obtain information on the orientation of the different secondary structures is certainly one of the greatest advantages of ATR-FTIR spectroscopy. Information on the orientation of the dipole can be obtained by measuring the IR spectra with different polarized incident light (polarized parallel and perpendicular with respect to the plane of incidence). The accuracy of the results obtained with this method has been recently demonstrated with bacterial porins, the atomic structures of which are known (31). The same method was applied here to get information on the structure of two membrane proteins for which no high-resolution structures were available. For a quantitative analysis of the $\beta$-strand tilt, the dichroic ratio of the lipid $\nu(C=O)$ band was chosen as representative of a dichroic ratio that would correspond either to an unordered dipole or to a dipole oriented at the magic angle with respect to the Ge-plate normal. It is called below $R_{\text{ATRiso}}$. With the value of $R_{\text{ATRiso}}$ the film thickness and the electric field amplitudes were computed. This procedure assumes that the
orientation of the lipid \(\nu(C=O)\) transition dipole moment is closed to the magic angle (54.7°). In fact, NMR and x-ray studies carried out on several phospholipids have suggested that the average orientation of the two fatty acyl chain \(\nu(C=O)\) transition dipole moments are close to the magic angle (44). The validity to determine \(R^{\text{ATRiso}}\) from the lipid \(\nu(C=O)\) band of polarized ATR-FTIR spectra has been demonstrated recently (45).

In this work, we calculated the orientation of the main secondary structure, \(i.e.\) anti-parallel \(\beta\)-strands, from the experimentally obtained dichroic ratio of the \(\beta\)-sheet component of the amide I band and the dichroic ratio of the amide II band. Our results show that the \(\beta\)-strands of VDAC and the OmpF porin are tilted at a very similar angle with respect to the barrel axis (\(\beta = 45\) to 47°, Table II). This result was obtained from expressions derived for regular \(\beta\)-barrels, which have an axial distribution of transition dipole moments around the barrel symmetry axis. It can be compared with the topology of planar \(\beta\)-sheets which display non-axial symmetry and for which the orientation of the \(\beta\)-strands can be calculated according to Marsh (29). Applying this approach to the dichroic ratios given in Table II we obtain a tilt angle of the \(\beta\)-strands \(\beta = 45\)° for both the two VDAC isoforms and the OmpF porin. Regarding OmpF, these results are in very good agreement with the value derived from the crystal structure (\(\sim 45\)°) where 11 strands are tilted at about 35° and 5 strands exhibit a more oblique tilt (21).

The structural geometries of a regular \(\beta\)-barrel are mainly determined by the number, \(n\), of \(\beta\)-strands and the tilt angle \(\beta\) of the \(\beta\)-strands (46). The radius, \(R\), of a cylindrical barrel is related to the strand tilt by,

\[
R = \frac{d}{2 \sin(n/2 \cos \beta)} \quad (\text{Eq. 3})
\]

where \(d = 0.472\) nm is the distance between two residues in a \(\beta\)-strand. From the knowledge of the barrel diameter and the mean orientation of the \(\beta\)-strands determined here by polarized ATR-FTIR, the number of \(\beta\)-strands in VDAC can be calculated. A diameter of VDAC of 3.7 nm has been determined by electron microscopy studies of yeast VDAC two-dimensional crystals and from low-resolution crystals of human VDAC (18, 19). Applying Equation 3 to this diameter and a mean \(\beta\)-strand tilt of 46° to 47° results in a barrel that is formed from 17 \(\beta\)-strands. Because \(\beta\)-barrels have an even number of \(\beta\)-strands VDAC could have either 16 or 18 \(\beta\)-strands (a tilt of 48° would give 16 \(\beta\)-strands and 44° 18 \(\beta\)-strands). Secondary structure predictions have suggested both, 16 and 18 \(\beta\)-strands (14, 47). The 16 (or 18) \(\beta\)-strands would have a mean length of approximately 9 (or 8) residues per strand, when 50 to 53% of the total residues (\(\sim 280\)) are in \(\beta\)-sheet conformation. From the strand tilt determined for OmpF (45°) and a mean diameter of 3.4 nm 16 \(\beta\)-strands are calculated which corresponds to that observed in the crystal structure.

Apparently, the VDAC \(\beta\)-barrels are somewhat more inclined in the lipid membrane than the OmpF barrels because the order parameters determined for the VDAC isoforms are significantly smaller than the one measured for OmpF. Assuming a similar ordering of the membrane in both systems, it can be calculated that the VDAC barrels are tilted at a higher angle than the OmpF barrel.

Hydrogen/deuterium exchange has long been used for the analysis of protein structure and dynamics. The H/D exchange data contain information regarding the strength of H-bonding, the solvent accessibility, and dynamic structure of proteins. Because the exchange of only the amide protons is monitored by IR spectroscopy, the exchange data are directly proportional to the number of amino acid residues in the protein. The exchange data suggest that the amide protons of VDAC and OmpF can be grouped in three populations with different time kinetics corresponding to fast, intermediate and slow exchange rates. Analysis of the data provide evidence that about 69% of the total OmpF amide protons exhibit a slow exchange rate, albeit 2.4 times faster than the OmpF amide protons. In contrast, the proportion of the amide protons that has a fast exchange rate is larger in
VDAC (30%) than in OmpF (17%). The exchange proceeded to a larger extent in VDAC than in OmpF. More than 60% of the VDAC amide protons were exchanged after 10 h compared to only 35% of the OmpF amide protons. Since both VDAC and OmpF form water filled pores, solvent accessibility should not be a limiting factor for the H/D exchange. In fact, it has been shown that high exchange rates correlates with the occurrence of water filled pores in a K⁺-channel and the aquaporin CHIP28 (48, 49). Apparently, strong H bonding causes the low exchange in OmpF whereas the exchange data indicate that less strong H bonding may occur in VDAC. When compared with the bacterial porin, a less stable sheet structure can be correlated with shorter strands in agreement with the first, the determination of the number and mean length of the strands in the barrel (see above) and second, the higher frequency of the sheet contribution to the amide I band as discussed above.

In conclusion, we show in this report that the two VDAC isoforms from P. vulgaris have a very similar secondary structure and orientation of the β-sheet and furthermore, their H/D exchange is undistinguishable. Taken together, this suggests that the two isoforms have a very similar overall structure. Furthermore, our data provide an experimental support to a general structural organization of both VDAC isoforms similar to the bacterial porin, a less stable sheet structure can be correlated with shorter strands in agreement with the first, the determination of the number and mean length of the strands in the barrel (see above) and second, the higher frequency of the sheet contribution to the amide I band as discussed above.

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