In Situ FTIR ATR Spectroscopy of the Preparation of an Oriented Monomolecular Film of Porin Omp32 on an Internal Reflecting Element by Dialysis

Michael Schwarzott,‡ Harald Engelhardt,‡ Thomas Klühspies,‡ Dieter Baurecht,† Dieter Naumann,§ and Urs P. Fringeli*†‡

Institute of Physical Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria, Max-Planck Institut für Biochemie, Am Klopferspitz 18, W-8033 Martinsried, Germany, and Robert Koch-Institut, P34, Norderer 20, 13353 Berlin, Germany

Received January 29, 2003. In Final Form: June 4, 2003

The development of a reproducible technique for the preparation of oriented porin Omp32 monolayers on an attenuated total reflection (ATR) internal reflection element (IRE) was the principal aim of this work. A procedure earlier applied for two-dimensional crystallization of membrane proteins [Paul, A.; Engelhardt, H.; Jakubowski, U.; Baumeyer, W. Biophys. J. 1992, 61, 172–188] could be successfully adapted. Layer formation was performed in a spectroscopic dialysis cell in the presence of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) micelles by the removal of the detergent (n-octylpolyoxyethylene, OPOE). This setup enabled in situ monitoring of the monolayer formation by means of the Fourier transform infrared (FTIR) single-beam sample-reference ATR technique. The time course of OPOE and sodium azide extraction by dialysis and the formation of the protein/lipid layer on top of the IRE was evaluated from corresponding time-dependent absorbance changes in the IR spectra. Both OPOE and azide feature first-order kinetics, whereas the synchronous adsorption of porin to the Ge IRE resulted in a sigmoidal time behavior. At the beginning of the dialysis, the concentration of OPOE was just above the critical micellar concentration (cmc) which is about 0.25%. As the cmc was reached by dialysis, Omp32 adsorption was accelerated, reaching saturation after about 5 h. Quantitative analysis of the Omp32/lipid layer gave strong evidence for a well-ordered monolayer with the barrel axis of the Omp32 trimer being approximately oriented perpendicular to the supporting IRE. Two to six DMPC molecules were detected per Omp32 monomer.

Introduction

Gram-negative bacteria are characterized by an outer membrane which serves as an effective protection against noxious compounds. The uptake of nutrients and ions is enabled by the outer membrane proteins referred to as porins. Porins are divided into two classes, the unspecific general porins, which build up unspecific diffusion pores, and those which form substrate-specific channels (for reviews, see refs 1–3). The general porins allow the transport of solutes up to a molecular mass of ~600 Da by passive diffusion,4 but they often show a selectivity for cations or anions, for example, OmpF and PhoE of Escherichia coli.5 The Omp32 from Deftia acidovorans (formerly Comamonas acidovorans) is a strongly anion selective porin.6 The three-dimensional structure was recently determined by X-ray crystallography at 0.21 nm resolution.7 As is typical for bacterial outer membrane proteins, Omp32 has a β-barrel structure. A monomer is built up of 16 amphipathic antiparallel β-strands connected by seven short periplasmic and eight external loops resulting in a molecular mass of 34.7 kDa. Each monomer of Omp32 forms a complex with a 5.8 kDa peptide. Porin and peptide together form a funnel-like homotrimer where the peptides are located close to the trimer axis at the periplasmic side. It is assumed that the peptides are involved in linking the outer membrane with the cell wall peptidoglycan.8 One of the external loops (L3) folds back into the center of the channel and determines together with a small protrusion (P1 of β strand j2) a particularly narrow constriction zone. A cluster of three charged arginine residues in this constriction in conjunction with further arginine and lysine residues at the external and periplasmic pore entrances creates a large positive electric potential within the channel.9 This is suggested to represent the selectivity filter of the porin. Omp32 shows voltage-dependent closing when incorporated in planar lipid bilayers.8 This kind of voltage gating means the enhanced probability of closed states of porin channels when a positive or negative electric potential exceeding a certain threshold value is applied. This gating phenomenon appears to be a general feature of β-barrel outer membrane proteins (for reviews, see refs 9 and 10). A recent study has shown the influence of charged amino acids within the constriction zone of OmpF on voltage sensing and conductance.11 It was found that an increase

* To whom correspondence should be addressed. Phone: +43-1-4277 525 30. Fax: +43-1-4277 9525. E-mail: urs.peter.fringeli@univie.ac.at.
† University of Vienna.
‡ Max-Planck Institut für Biochemie.
§ Robert Koch-Institut.

in the charge density increases the ion flow, whereas the effects on voltage gating could not be correlated with the charge state of the pore constriction. Conformational changes of extracellular loops of OmpF in response to an applied electric potential, which led to an occlusion of the pore entrance, were observed by atomic force microscopy measurements. This possible mechanism of voltage gating was supported by a study of channel conductance in a planar lipid bilayer of a porin from Haemophilus influenzae type b, where single mutations of amino acids in an extracellular loop had changed the threshold value for channel closing. To verify this mechanism, an experimental approach is required, which is able to monitor conformational changes. Attenuated total reflection (ATR) Fourier transform infrared (FTIR) difference spectroscopic methods are capable of detecting changes in protein structure. Moreover, modulation spectroscopy enables access to a kinetic analysis of the sample response of a varying external parameter (e.g., electric field). However, independently of the chosen ATR FTIR method, it is a prerequisite to achieve a reproducible technique of protein immobilization on the internal reflection element (IRE).

In this paper, we present a method for Omp32 monolayer preparation which was adapted from two-dimensional crystallization of membrane proteins in the presence of phospholipids, as used for example earlier for sample preparation in electron microscopy. As a consequence of slow removal of the detergent from a mixture of solubilized porin and lipid micelles by dialysis, the protein reconstituted into the lipid matrix. It formed layers with different symmetries and dimensions of the unit cell, depending on the experimental conditions applied. Relevant parameters turned out to be the lipid-to-protein ratio, the temperature, and the nature of lipids or detergents. We have used an ATR flow-through dialysis cell which allowed dialysis of the Omp32 and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) suspension, monitoring the process in situ by FTIR ATR spectroscopy. Our conditions were similar to those described in ref 17.

### Materials and Methods

**Materials.** DMPC was purchased from Fluka Chemie GmbH (Buchs, Switzerland), deuterium-labeled 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DM-δ67-PC, all protons except the 2,0-δ9-PO3) from Bachem AG (Bubendorf, Switzerland). Other chemicals were of analytical grade and obtained from Fluka Chemie GmbH. All chemicals were used without further purification. Porin Omp32 was extracted by solubilization of the porin and lipid with a lipid-to-protein ratio (L/P) (mol/mol) of 9.2 per volume, which was bordered by the IRE and the dialysis membrane, while the outer compartment of the dialysis membrane was immersed in the sample and the cell back-wall. Both compartments are hydromagnetically optimized for flow-through. In this experiment, the dialysis membrane had a pore size of 5 kDa (Messenger Membrane Systems AG, Zürich, Switzerland).

**Dialysis.** An aliquot of a solution of DMPC or DM-δ67-PC in chloroform was dried in a nitrogen stream and then dissolved in the Omp32 buffer solution by vortexing. The solution contained 4.0 mg/mL Omp32, 9.8 mg/mL of DMPC (calculated as a mol mass of 40.5 kDa). This mixture of detergent-solubilized porin and lipid with a lipid-to-protein ratio (L/P) (mol/mol) of 9.2 ± 2.3 (DMPC) and of 8.3 ± 2.1 (mol/mol) (DM-δ67-PC), respectively, was filled into the inner compartment of the sample side of the dialysis cell. This compartment is in direct contact with the IRE. Then the tubes at the inlet and outlet were clamped at the entrance of the cell. The temperature of the cell was set to 35 ± 0.5 °C, that is, well above the transition temperature of DMPC (−24 °C from ref 20). A 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl was used as the dialysis buffer and was filtered into the reference side of the cell. The buffer was degassed and thermostated before it was pumped through the outer chamber of the sample side of the dialysis cell. The pump speed of the dialysate was 0.28 mL/min. SBSR absorbance spectra were recorded in situ during the dialysis process, which was stopped when no additional spectral changes were noticeable within at least 1 h of observation. A SBSR background spectrum of the phosphate buffer filled into both the sample and the reference side of the IRE, respectively. All spectra were scanned at 4 cm⁻¹ resolution with a zero filling factor of 4 and a Blackman Harris 3 term apodization. The spectrometer was purged with dry and carbon dioxide free air. A germanium trapezoid (54 × 30 × 1.5 mm³) with an angle of incidence θ = 45° was used as the IRE. The mean number of active internal reflections in the IRE was N = 14.1 throughout all experiments. Spectra were recorded with parallel (pp) and perpendicular polarized (vp) incident infrared light, and 500 – 1000 scans were accumulated to achieve the desired signal-to-noise ratio. The IRE (Ge plate) was polished by means of 0.25 μm diamond paste on a pellon cloth (Logitech Ltd., Old Kilpatrick, Neerach, Switzerland) was used. The attachment was equipped with a liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector. In SBSR measurements, the infrared beam propagates alternately through the upper and lower half of the IRE with a short mutual time delay. One-half of the ATR multiple IRE is used for the sample (S), and the other one for the reference (R). SBSR absorbance spectra are calculated from corresponding single-channel spectra recorded with a mirror passing through the sample side and the reference side of the IRE, respectively.

**Materials and Methods.**

**Materials.** DMPC was purchased from Fluka Chemie GmbH (Buchs, Switzerland), deuterium-labeled 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DM-δ67-PC, all protons except the 2,0-δ9-PO3) from Bachem AG (Bubendorf, Switzerland). Other chemicals were of analytical grade and obtained from Fluka Chemie GmbH. All chemicals were used without further purification. Porin Omp32 was extracted by solubilization of the porin and lipid with a lipid-to-protein ratio (L/P) (mol/mol) of 9.2 per volume, which was bordered by the IRE and the dialysis membrane, while the outer compartment of the dialysis membrane was bordered by the sample side and the cell back-wall. Both compartments are hydromagnetically optimized for flow-through. In this experiment, the dialysis membrane had a pore size of 5 kDa (Messenger Membrane Systems AG, Zürich, Switzerland).

**Dialysis.** An aliquot of a solution of DMPC or DM-δ67-PC in chloroform was dried in a nitrogen stream and then dissolved in the Omp32 buffer solution by vortexing. The solution contained 4.0 mg/mL Omp32, 9.8 mg/mL of DMPC (calculated as a mol mass of 40.5 kDa). This mixture of detergent-solubilized porin and lipid with a lipid-to-protein ratio (L/P) (mol/mol) of 9.2 ± 2.3 (DMPC) and of 8.3 ± 2.1 (mol/mol) (DM-δ67-PC), respectively, was filled into the inner compartment of the sample side of the dialysis cell. This compartment is in direct contact with the IRE. Then the tubes at the inlet and outlet were clamped at the entrance of the cell. The temperature of the cell was set to 35 ± 0.5 °C, that is, well above the transition temperature of DMPC (−24 °C from ref 20). A 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl was used as the dialysis buffer and was filtered into the reference side of the cell. The buffer was degassed and thermostated before it was pumped through the outer chamber of the sample side of the dialysis cell. The pump speed of the dialysate was 0.28 mL/min. SBSR absorbance spectra were recorded in situ during the dialysis process, which was stopped when no additional spectral changes were noticeable within at least 1 h of observation. A SBSR background spectrum of the phosphate buffer filled into both the sample and the reference sides of the IRE was subtracted from all SBSR spectra in order to get enhanced background compensation by compensating inhomogeneties resulting predominantly from the IRE and the sensitive area of the MCT detector element.

**H/D Exchange.** After porin had adsorbed onto the Ge IRE, the temperature of the dialysis cell was reduced to 23 ± 0.5 °C.
Table 1. Optical and Spectroscopic Parameters for Quantification of the Surface Concentration of Omp32, DMPC, and DM-d67-PC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Omp32, amide I'</th>
<th>Omp32, ν(NH)</th>
<th>DMPC, ν(CH2)</th>
<th>DM-d67-PC, ν(CH2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n_0</td>
<td>4.0 ± 0</td>
<td>4.0 ± 0</td>
<td>4.0 ± 0</td>
<td>4.0 ± 0</td>
</tr>
<tr>
<td>n_1</td>
<td>1.4 ± 0.06</td>
<td>1.4 ± 0.06</td>
<td>1.45 ± 0.06</td>
<td>1.45 ± 0.06</td>
</tr>
<tr>
<td>n_2</td>
<td>1.32 ± 0.06</td>
<td>1.27 ± 0.06</td>
<td>1.22 ± 0.06</td>
<td>1.38 ± 0.06</td>
</tr>
<tr>
<td>θ/deg</td>
<td>45 ± 15</td>
<td>45 ± 15</td>
<td>45 ± 1.5</td>
<td>45 ± 1.5</td>
</tr>
<tr>
<td>f(0)/ν(cm⁻¹mol⁻¹)</td>
<td>2.74 ± 10⁷</td>
<td>2.09 ± 10⁶</td>
<td>5.7 ± 10⁴</td>
<td>5.9 ± 10⁵</td>
</tr>
<tr>
<td>Δν/ν(cm⁻¹)</td>
<td>1706–1595</td>
<td>3400–3200</td>
<td>2869–2830</td>
<td>2131–2045</td>
</tr>
<tr>
<td>f_max/cm⁻¹g</td>
<td>1650</td>
<td>3280</td>
<td>2852</td>
<td>2093</td>
</tr>
<tr>
<td>ν(CH2)</td>
<td>1384</td>
<td>364</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>ν(CH)</td>
<td>14.1 ± 1</td>
<td>14.1 ± 1</td>
<td>14.1 ± 1</td>
<td>14.1 ± 1</td>
</tr>
</tbody>
</table>


(room temperature), the buffer was removed, and the dialysis cover of the cell was replaced by a standard SBSR flow-through cap. Both sides of the cell were refilled immediately with degassed buffer of room temperature, so that the porin film remained hydrated during this process. Then the buffer in both compartments (R and S) of the SBSR cell was replaced by 150 mM NaCl in D₂O, initiating the H/D exchange. Time-resolved measurements were started immediately after H₂O buffer replacement.

At the end of this experiment, the porin film was removed by means of a 0.3 M SDS solution containing 0.1 M EDTA to enable the measurement of background SBSR spectra in the D₂O environment.

Monofilm Preparation. To calculate the lipid coverage in the dialysis experiment, dry DMPC and DM-d67-PC monolayers were prepared on an IRE by means of the Langmuir–Blodgett (LB) technique as described for 1,2-dipalmitoyl-snglycero-3-phosphoric acid (DPPA)²³ by means of a film balance (Nima Technology Ltd., Science Park, Coventry, U.K.). The surface pressure was 30 ± 0.2 mN/m during film transfer at room temperature, so that the porin film remained hydrated during this process. Then the buffer in both compartments (R and S) of the SBSR cell was replaced by 150 mM NaCl in D₂O, initiating the H/D exchange. Time-resolved measurements were started immediately after H₂O buffer replacement.

Theoretical Section

Orientation Measurements. Orientation measurements require independent spectra of the sample by means of parallel and perpendicular polarized incident light. To get rid of physical and molecular constants, such as the magnitude of the transition moment, the so-called dichroic ratio (i) was determined by means of integrated absorbances of measured spectra with parallel and perpendicular polarized incident light. Peak absorbances may also be used instead of integrated absorbances.

By means of the real parts of the refractive indices of the IRE, the thin film and the bulk medium, as well as the angle of incidence, one obtains the relative electric field components E_x(i), E_y(i), and E_z(i) at a distinct wavenumber of a thin film in contact with a bulk rarer medium according to Harrick’s approximation of Fresnel’s equations.²⁷,²⁸ This so-called thin film approximation is valid when the film thickness is very small compared to the depth of penetration and in the case of a weakly absorbing sample.²⁹ Both conditions are fulfilled for a monomolecular layer of biological material. Assuming a LCU, that is, an uniaxial arrangement of the sample, one obtains for the dichroic ratio the following expression:

\[ R = \frac{\int A_{\phi}(i) \, di}{\int A_{\psi}(i) \, di} = \frac{E_x^2}{E_y^2} + 2 \frac{E_z^2 \cos^2(\Theta)}{E_y^2 (1 - \cos^2(\Theta))} \]

(Note that for the sake of simplicity, the dependence of the dichroic ratio and the relative electric field components on the wavenumber (i) is not explicitly indicated.)

\((\cos^2(\Theta))\) denotes the average over the mean squares of the cosines of the angles between the transition moments of a given vibration and the z-axis of the laboratory coordinate system, which is fixed to the IRE. The xy-plane

is parallel to the surface of the IRE, and the x-axis is parallel to the direction of light propagation. The z-axis is perpendicular to the IRE surface.

Solving eq 1 for the mean square cosine results in

\[
\langle \cos^2 \Theta \rangle = \frac{E_x^2 - RE_y^2}{E_x^2 - RE_y^2 - 2E_z^2}
\]

(2)

Three special cases of molecular orientation should be mentioned here, the isotropic arrangement of transition dipole moments, resulting in \( \langle \cos^2 \Theta \rangle_{iso} = \frac{1}{3} \), the perfect alignment of the transition dipole moments parallel to the surface of the IRE (xy-plane), resulting in \( \langle \cos^2 \Theta \rangle_{xy} = 0 \), and perfect alignment of transition moments along the normal to the IRE (z-axis), resulting in \( \langle \cos^2 \Theta \rangle_{z} = 1 \), respectively. As can be derived from eq 1, the corresponding dichroic ratios are

\[
R_{iso} = \frac{E_x^2 + E_z^2}{E_y^2} \quad R_{xy} = \frac{E_x^2}{E_y^2} \quad R_z = \infty
\]

(3)

Dichroic difference spectra \( D^* \) offer a fast and easy way to get orientational information. A \( D^* \) spectrum is defined as the weighted difference spectrum between the absorbance spectra measured with parallel and perpendicular polarized incident light as given in eq 4.

\[
D^*(\vec{\gamma}) = A_{pp}(\vec{\gamma}) - R_{iso}A_{vp}(\vec{\gamma})
\]

(4)

The weighting factor \( R_{iso} \) has to be calculated as shown above and denotes the dichroic ratio of an isotropic sample. Contrary to transmission spectroscopy, \( R_{iso} \) differs from unity and has to be calculated in each case depending on the optical constants of the sample. Consequently, the dichroic difference spectrum as defined by eq 4 results in a horizontal line if the sample is isotropic or the respective mean transition moment assumes the so-called magic angle of \( \Theta = 54.74^\circ \) (uniangular orientation) with the axis normal to the IRE surface. A positive \( D^* \) band indicates predominant alignment of transition moments of a given vibration in the direction of the z-axis, that is, \( \Theta < 54.74^\circ \), while a negative \( D^* \) band is significant for predominant alignment of the transition moments parallel to the reflecting surface of the IRE (xy-plane), that is, assuming a mean angle \( \Theta > 54.74^\circ \) with the z-axis.

**Determination of Surface Concentration.** The volume concentration \( c \) and the surface concentration \( \Gamma \) are related to each other via the thickness of the sample \( d \) and Lambert–Beer’s law by

\[
c = \Gamma \cdot \frac{d}{N \cdot \nu \cdot d_{e,pp} / \epsilon(\gamma) \cdot d_{e,pp}}
\]

(5)

where, as before, \( \int A_{vp}(\vec{\gamma}) \, d\vec{\gamma} \) denotes the integrated absorbance of a distinct absorption band obtained by a measurement with perpendicular polarized light. \( N \) and \( \nu \) are the mean number of the active internal reflections and the number of equal functional groups (e.g., amide groups) per molecule. \( \int \epsilon(\gamma) \, d\vec{\gamma} \) denotes the decadic integrated molar absorption coefficient of this band. The effective thickness of an arbitrarily oriented sample per internal reflection is denoted by \( d_{e,pp} \). It indicates the hypothetical sample thickness required for a transmission experiment in order to give the same absorbance as obtained with the real ATR measurement, thus enabling the use of Lambert–Beer’s law for quantitative ATR spectroscopy. The corresponding analytical expressions for perpendicular and parallel polarized light are composed of the axial contributions which depend on the corresponding effective thickness of an isotropic sample as well as on the mean square cosine according to eqs 6 and 7.

\[
d_{e,pp} = d_{e,x} + d_{e,z} = \frac{3}{2}(1 - \langle \cos^2 \Theta \rangle) d_{e,iso}^2 + 3\langle \cos^2 \Theta \rangle d_{e,iso}^2
\]

(6)

\[
d_{e,vp} = d_{e,y} = \frac{3}{2}(1 - \langle \cos^2 \Theta \rangle) d_{e,iso}^2
\]

(7)

According to Harrick’s thin film approximation, the axial effective thickness of an isotropic sample \( d_{e,iso}^2 \) is proportional to the real sample thickness \( d \), thus enabling the definition of the relative effective thickness, depending only on optical parameters.

\[
d_{e,iso} = \frac{n_2}{n_1 \cos \theta} E_{\gamma^2}
\]

(8)

\( n_1 \) is the refractive index of the IRE, \( n_2 \) is the refractive index of the film, \( \theta \) is the angle of incidence, and \( x, y, z \), respectively.

Using the relative isotropic effective thickness \( \Gamma = d_{e,iso} / d_{e,pp} \) and inserting eq 2 for the mean square cosine \( \langle \cos^2 \Theta \rangle \), one obtains the following expression for the surface concentration of a substance in a thin film assuming a liquid crystalline ultrastructure:

\[
\Gamma = \frac{\int A_{vp}(\vec{\gamma}) \, d\vec{\gamma}}{3 N \cdot \nu \cdot d_{e,pp} / \epsilon(\gamma) \cdot d_{e,pp} \left[ \frac{2 - E_x^2 + E_z^2}{E_x^2 + E_z^2} \right]}
\]

(9)

The surface concentration \( \Gamma \) as given in eq 9 may be understood as the projection of the molecules in the volume defined by the unit area and the height \( d \) (real sample thickness).

In a recent review article, it was shown that eqs 1–9, which are based on the thin film and the weak absorber approximation, are in excellent agreement with straightforward accurate ATR data analysis even for many bulk organic and inorganic media.

**Error Analysis.** The uncertainties indicated in this paper are all based on a straightforward error propagation calculation. Analytical expressions for partial derivatives have been evaluated by means of the Symbolic Mathematical Toolbox of MATLAB, whereas the final numeric calculation of overall uncertainties has been performed by means of the standard MATLAB software. The uncertainties of the input parameters are presented in Table 1. They correspond to approximately a 95% limit of confidence.

**Results**

**Preparation of Oriented Omp32/Lipid Layers.** The mixture of Omp32 and deuterated DM–d67–PC (or DMPC


in other experiments) solubilized in 0.5% OPOE was filled into the inner compartment of the sample (S) cuvette of the dialysis SBSR cell. The outer chamber contained pure dialysis buffer, which was not moved during the first measurement started immediately after filling the S-cuvette. The resulting spectrum at t = 0 h is shown in Figure 1. Since a SBSR measurement with 1000 scans per sample takes about 20 min, this spectrum reflects an average state of the sample of the first 10 min of dialysis. The band at 2048 cm⁻¹ results from the N₃ stretching (ν viewPager(N₃)) of the azide ion. The strong peak at 1082 cm⁻¹ and the weaker one at 990 cm⁻¹ are vibrations of the detergent OPOE. Small absorbances around 2900 cm⁻¹ originated from overlapping vibrations of the methylene groups of detergent and porin, respectively. The two prominent bands in the range between 1700 and 1500 cm⁻¹ result from the amide I and amide II vibrations of the protein. The buffer in this SBSR measurement was almost completely compensated, leaving only a small broad band from ν(OH) stretching of H₂O between 3700 and 3600 cm⁻¹. After the first spectrum was acquired, the dialysis buffer was pumped through the outer part of the S-cuvette while the R-cuvette was capped without circulation. The following spectra were recorded at a constant flow rate of buffer of 0.28 ml/min. Typical for the progress of the diffusion/desorption process is the fast decrease of the intensity of the azide band and the slower vanishing of OPOE bands. This behavior was expected because of the much smaller size of the N₃⁻ ion compared to the detergent OPOE. The evanescent electric field of the infrared light decreases exponentially with the distance from the surface of the IRE. Therefore, the absorbance of a substance increases with decreasing distance to the surface of the IRE and reaches a maximum at the surface. Therefore, the appearance of increasing protein bands indicates unambiguously adsorption of Omp32 to the IRE.

The development of the shape of the amide I band between 1690 and 1600 cm⁻¹ shows an intense maximum at 1630 cm⁻¹ and a weak shoulder at 1694 cm⁻¹. This is characteristic of a protein rich in antiparallel β pleated sheet structure, an observation which is consistent with data from X-ray crystallography, reporting that 52% of the amino acids are involved in β strands taking the 54 amino acid peptide into account. Parallel to the increase of amide I and amide II bands, a pronounced intensity decrease for ν(OH) occurs. This observation can be explained by the displacement of water in the immediate vicinity of the IRE surface by the growing Omp32 layer on the ATR element in the sample cuvette. The spectrum at the bottom of Figure 1 was measured after the flow-through of the dialysis buffer was stopped (18.7 h). After this time, no further increase of the protein bands could be observed within 1 h. There are weak but unambiguously detectable bands resulting from the stretching vibrations ν viewPager(CD₂)(CD₂) and ν viewPager(CD₂) at 2196 and 2093 cm⁻¹ resulting from incorporated deuterated DMPC. Spectra were separated by an absorbance shift of 0.04 AU. Note that the small azide ion (2048 cm⁻¹) is faster extracted than the detergent OPOE (1000 cm⁻¹ region). The formation of the porin layer is most clearly documented by the appearance of the amide I and II bands in the range between 1700 and 1500 cm⁻¹. Ge IRE; angle of incidence θ = 45°; number of active internal reflections N = 14.1 ± 1.

The time-dependent behavior of the components as shown in Figure 2 was evaluated from time-resolved difference spectra, taking the spectrum at the end of dialysis as a reference. The normalized integrated absorbances (band areas) of the azide ion at 2048 cm⁻¹, OPOE at 1082 cm⁻¹, and the protein at 1542 cm⁻¹ were used. The amide I band overlaps by the bending vibration of bound water, displaced bulk water, and possible slight incompensations of bulk water. Normalization was performed to enable easier comparison of the curves. The largest value of a corresponding band area was set to 1, reflecting the maximum concentrations of 0.5% OPOE and 3 mM NaN₃, or the highest intensity of the amide II band at the end of dialysis, respectively. The decrease of NaN₃ and the detergent could be fitted by a
The adsorption of Omp32 was almost finished within the first hour of dialysis. The amide protons did not exchange within this time period. Consequently, they exist as a negative OH bending band at 1645 cm\(^{-1}\) masking the behavior of the amide I band. Furthermore, a decrease of approximately 50% of the Omp32 porin was found. We expect that washing of the adsorbed layer and the exchange of the cell cap are the main reasons for this loss, and since the remaining layer was absolutely stable, we suggest that the loss reflects the detachment of loosely bound materials on top of the stable layer of porin and lipid on the Ge plate. A similar behavior was observed in the course of lipid bilayer preparation by means of the LB/vesicle method.\(^{21}\)

**Sensing Amide Proton Accessibility by Deuterium (H–D) Exchange Measurements.** Hydrogen–deuterium (H–D) exchange was initiated by replacing the H\(_2\)O phosphate buffer by a 0.15 M NaCl solution in D\(_2\)O. SBSR spectra of the porin layer recorded with polarized light are shown in Figure 3B. The negative water bending vibration \(\delta\) (H\(_2\)O) at 1645 cm\(^{-1}\) which overlapped the amide I band (Figure 3A) appears now as \(\delta\) (D\(_2\)O) at 1206 cm\(^{-1}\). As a consequence, the intensity of the amide I' band at 1635 cm\(^{-1}\) (Figure 3B) is undisturbed by the solvent and reflects the whole amount of the adsorbed Omp32. The amide I' mode, mainly consisting of the ND\(_2\) in-plane bending vibration, is shifted to 1455 cm\(^{-1}\). A considerable part of the amide protons did not exchange within the 17 h of observation. This is unambiguously documented by the remaining NH stretching band \(\nu(NH)\) at 3280 cm\(^{-1}\) resulting from protonated amide groups. Complete replacement of the H\(_2\)O buffer by D\(_2\)O was achieved within 7 min using a flow rate of 0.28 mL/min. After about 45 min, the D\(_2\)O flow-through was stopped. During the following 17–19 h, a very slow continuation of the exchange of the amide protons could be observed as shown for example for the porin/DM-d67-PC layer spectra in Figure 4. Calculating the surface concentration of Omp32 after about 18 h by means of the remaining \(\nu(NH)\) band at 3280 cm\(^{-1}\) resulted in a value which was about 57% smaller than that obtained via the amide I' band (see Table 2), suggesting that 43% of the amide protons did not exchange within this time period. Obviously, 43% of the amino acid residues of Omp32 belong to rather rigid structural regions or are hidden within the membrane.
protein, inaccessible to D2O. Moreover, within the first 8 min 49% of amide protons exchanged, followed by an additional exchange of about 4% between 8 and 45 min. Between 45 min and about 18 h, it was found that a further 4% of the amide protons were exchanged. It follows that about 8% of the secondary structure exhibits medium flexibility whereas 49% of the amino acids of Omp32 undergo a fast H–D exchange. Consequently, about one-half of the secondary structure exhibits a sufficient flexibility for H–D exchange or a good accessibility to water molecules.

These results are in good agreement with a study of the H–D exchange of a hydrated film of porin OmpF, where kinetics with a fast, an intermediate, and a very slow exchange rate exhibiting time constants in the range of 1.3 min, 28 min, and 94 h have been reported. In the region of the amide I band at ~1540 cm⁻¹, there exist additional absorption bands of amine acid sidechains. We assigned tentatively the band at 1583 cm⁻¹ to the asymmetric stretching vibration νas(CO⁻) of carboxylate groups (aspartic and glutamic acid residues) and the band at 1515 cm⁻¹ to an aromatic ring vibration of tyrosine, consisting of ν(C–C)ring and δ(CH). Molar lipid-to-protein ratio after porin monolayer formation at the IRE surface.

Table 2. Quantitative Analysis of Experimental Data

<table>
<thead>
<tr>
<th>Omp32/DMPC</th>
<th>R</th>
<th>Omp32/DM-67-PC</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>j(amide I)</td>
<td>2.349 ± 0.110</td>
<td>1.98 ± 0.13</td>
<td>2.164 ± 0.100</td>
</tr>
<tr>
<td>j(amide II)</td>
<td>1.187 ± 0.055</td>
<td>1.065 ± 0.050</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>j(Omp32)</td>
<td>7.78 × 10⁻¹³ ± 1.40 × 10⁻¹²</td>
<td>7.012 ± 1.29 × 10⁻¹²</td>
<td>1.31 ± 0.84</td>
</tr>
<tr>
<td>j(Omp32)</td>
<td>0.0134 ± 0.0020</td>
<td>0.0102 ± 0.0040</td>
<td>0.0078 ± 0.040</td>
</tr>
<tr>
<td>j(Omp32)</td>
<td>0.0129 ± 0.0019</td>
<td>0.0102 ± 0.0040</td>
<td>0.0078 ± 0.040</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>3.8 × 10⁻¹¹ ± 0.7 × 10⁻¹¹</td>
<td>2.6 × 10⁻¹¹ ± 1.6 × 10⁻¹¹</td>
<td>2.01 ± 0.14</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>0.769 ± 0.039</td>
<td>0.714 ± 0.036</td>
<td>0.714 ± 0.036</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>0.397 ± 0.020</td>
<td>0.355 ± 0.018</td>
<td>0.332 ± 10⁻¹²</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>3.60 ± 10⁻¹² ± 6.45 × 10⁻¹³</td>
<td>2.32 ± 10⁻¹² ± 6.01 × 10⁻¹³</td>
<td>2.01 ± 0.14</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>0.718 ± 0.035</td>
<td>0.714 ± 0.036</td>
<td>0.714 ± 0.036</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>0.377 ± 0.019</td>
<td>0.332 ± 0.016</td>
<td>0.332 ± 0.016</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>3.38 × 10⁻¹² ± 6.03 × 10⁻¹³</td>
<td>3.05 × 10⁻¹² ± 5.56 × 10⁻¹³</td>
<td>2.01 ± 0.14</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>0.85 ± 0.08</td>
<td>0.76 ± 0.08</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>9.2 ± 2.3</td>
<td>8.3 ± 2.1</td>
<td>8.3 ± 2.1</td>
</tr>
<tr>
<td>j(DMPC)</td>
<td>4.9 ± 1.3</td>
<td>3.7 ± 2.3</td>
<td>3.7 ± 2.3</td>
</tr>
</tbody>
</table>

a Integrated absorbance amide I, linear baseline 1706–1595 cm⁻¹. b Omp32 monomer surface concentration. c Integrated absorbance of ν(NH) (after 17 h contact with D2O), linear baseline 3400–3200 cm⁻¹. d Integrated absorbance of ν(NH) (after approximately 45 min contact with D2O), linear baseline 3400–3200 cm⁻¹. e Integrated absorbance of ν(NH) (after 17 h contact with D2O), linear baseline 3400–3200 cm⁻¹. f Integrated absorbance of ν(NH) (after 17 h contact with D2O), linear baseline 3400–3200 cm⁻¹. g Integrated absorbance of ν(NH) (after 8 min contact with D2O), linear baseline 3400–3200 cm⁻¹. h Integrated absorbance of ν(NH) (after 17 h contact with D2O), linear baseline 3400–3200 cm⁻¹.
which were produced by the conventional dialysis tech-

studies of 2D crystals from Omp32 (space group D*). The underlying model of a LCU, the transition moments of both conformational populations are assumed to exhibit a uniaxially ordered layer. This finding is consistent with a uniaxially ordered layer. This finding is consistent with 1635 cm\(^{-1}\) structure from an electron micrograph of this kind of membrane proteins could be successfully adapted for use with ATR IREs. The procedure described in this paper leads to well-oriented monolayers of Omp32, where each protein monomer is associated with 2-6 lipid molecules. The observation of a saturation in the course of Omp32 adsorption strongly suggests that a monolayer is formed (Figure 2). Moreover, distinct dichroic phenomena (Figure 5) give evidence for an Omp32/DMPC assembly forming a uniaxially ordered layer. This finding is consistent with electron microscopic data obtained from OmpF/DMPC layers prepared by the same method. The very slow increase of the amide II absorbance at elevated temperature (Figure 2) can be explained by the adsorption of loosely bound Omp32 aggregates to the stable monolayer, since these aggregates turned out to be easily eliminated by rinsing the flow-through cuvette with pure buffer, while the magnitude of amide II absorbance detected within the first 5 h remained stable.

Omp32/DMPC and Omp32/DM-67-PC layers resulted in a mean area per Omp32 trimer of 67 ± 13 nm\(^2\). This finding is in good agreement with electron microscopical studies of 2D crystals from Omp32 (space group p312), which were produced by the conventional dialysis tech-

Figure 5. Dichroic difference spectra \(D^* = A_{pp} - R_{iso}A_{vp}\) from the Omp32/DM-67-PC monolayer using the calculated isotropic dichroic ratio for the amide I region \(R_{iso} = 1.65\) and the associated limits of about 95% confidence \(R_{iso} = 1.40\) and \(R_{iso} = 1.90\), respectively. The underlying SBSR absorbance spectra \(A_{pp}, A_{vp}\) measured with parallel (pp) and perpendicular (vp) polarized incident light are shown on top (scaled down by a factor 0.3). For the ideal presentation, \(D^*\) with \(R_{iso} = 1.65\) is shifted by 5 mAU, \(D^*\) with \(R_{iso} = 1.40\) is shifted by 10 mAU, and \(A_{pp}\) and \(A_{vp}\) are shifted by 25 and 30 mAU, respectively. Optical parameters are as in Figure 4.

Figure 6. Correlation average of an electron micrograph from a two-dimensional crystal of isolated Omp32. The specimen was negatively stained with uranyl acetate. Black areas indicate pores and holes filled with stain, and bright areas indicate biological material, i.e., protein. The space group is p321 with a unit cell containing two trimers in opposite orientation with respect to the membrane plane. The symmetry was determined by decoration experiments. The unit cell is marked and has a size of \(a = b = 13.5\) nm, enclosing an angle of \(\gamma = 120^\circ\).
two-dimensional microcrystals with barrel axes near normal to the IRE surface. However, near 1623 cm\(^{-1}\) there is a distinct negative D\(^{+}\) band which could also be assigned to the intense low-frequency component of the antiparallel \(\beta\)-sheet. In this case, however, no obvious consistence with the suggested model would exist. Future experiments and theoretical considerations have to give more detailed information with respect to the assignment and the direction of transition moments of the vibrations detected at 1630 and 1623 cm\(^{-1}\).

In this paper, we have described a procedure enabling the preparation of a stable oriented monolayer of Omp32 on an ATR crystal. Therefore, a principal prerequisite for the study of electric field dependent channel gating by electric field modulated excitation spectroscopy is fulfilled. Experiments using a setup as presented in ref 38 are in progress.

**Acknowledgment.** This work was supported by a grant (Na 226/9-2) from the Deutsche Forschungsge-