In Situ Preparation and Electric Field Excitation of an Oriented Film of Porin Omp32 monitored by FTIR ATR Spectroscopy

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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. The general porins often show a selectivity to either cations or anions. The Omp32 from *Delftia acidovorans* is a strongly anion selective porin (1). The three dimensional structure was recently determined by X-ray crystallography at 0.21 nm resolution (Fig. 1).

Omp32 shows voltage dependent closing when incorporated in planar lipid bilayers. Conformational changes of extra-cellular loops of OmpF in response to an applied electric potential , which lead to an occlusion of the pore entrance, were observed by atomic force microscopy measurements (2). Now, an experimental approach is required, which is able to monitor conformational changes in situ. ATR FTIR difference spectroscopic methods are capable to detect 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) (3,4). For FTIR-ATR spectroscopy, it is a prerequisite to achieve a reproducible technique of protein immobilization on the internal reflection element (IRE).

Here we present a method for Omp32 monolayer preparation which was adapted from two-dimensional crystallization of membrane proteins in the presence of phospholipids. Preliminary results of FTIR modulation spectra obtained by modulated E-field excitation of the Omp32 monolayer are shown.

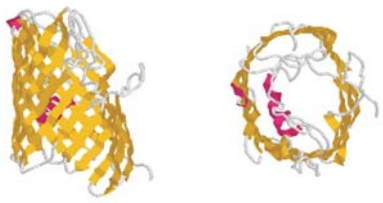

Fig. 1: Three dimensional structure of Omp32 monomer (7). As typically for bacterial outer membranes proteins, Omp32 has a -barrel structure. A monomer is built up of 16 amphipathic antiparallel strands connected by seven short periplasmic and eight external loops resulting in a molecular mass of 34.7 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. One of the external loops (L3) folds back into the center of the channel and determines together with a small protrusion (P1 of strand 2) 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 create a large positive electric potential within the channel. This is suggested to represent the selectivity filter of the porin.

Materials and Methods

FTIR-ATR Spectroscopy. Bruker FTIR spectrometer (IFS-66 and IFS25) with a „lift-model" single beam sample reference (SBSR) mirror attachment (OPTISPEC, CH-8173 Neerach, Switzerland) were used. Spectra were recorded with parallel (pp) and perpendicular polarized (vp) incident infrared light. 500 - 1000 scans were accumulated to achieve the desired signal to noise ratio in stationary spectra.

Dialysis. A SBSR dialysis cell was used (OPTISPEC, CH-8173 Neerach, Switzerland) featuring an inner compartment of approximately 100 µL volume. An aliquot of a solution of DMPC or DM-d67-PC was dissolved in the Omp32 buffer solution containing 4±1 mg/mL Omp32. This mixture of detergent solubilizied porin and lipid was filled into the inner compartment of the sample side of the dialysis cell which is in direct contact with the IRE. A 10 mM phosphate buffer was used as dialysis buffer and was filled into the reference side of the cell. The dialysis membrane had a pore size of 5 kDa

SBSR absorbance spectra were recorded *in situ* during the dialysis process, which was stopped when no additional spectral changes were noticeable within at least one hour of observation.

H/D exchange. After porin had adsorbed onto the Ge IRE the buffer was removed and the dialysis cover of the cell was replaced by a standard SBSR flow through cap. Then the buffer in both compartments (R and S) of the SBSR cell was replaced by 150mM NaCl in D_2O initiating the H/D exchange.

Data analysis. Determination of the surface concentration of Omp32 and DMPC/DM-d67-PC were performed by means of equations derived with the thin film and weak absorber approximations as described in (5). Uniaxial orientation with the feature of liquid crystalline ultrastructure (LCU) was assumed for both, adsorbed Omp32 and DMPC as well as DM-d67-PC, respectively.

Electric field modulation spectroscopy. The oriented monolayer of Omp32 was transferred in an ATR electrochemical flow through cell which was filled with 1 mM NaCl in D_2O (Fig.5a). The applied electric stimulus was a sine wave with a period of 5 min and a peak to peak amplitude of 1 V. Synchronization of modulated excitation and measurement of time resolved FTIR is shown in Fig.5b. To enhance signal to noise ratio, 32 scans were measured for of a single time-resolved spectrum and additionally coaddition was done during successive modulation periods (e.g. 205 modulation periods leading to 6560 scans per time resolved spectrum were accumulated for the spectra shown in Fig.6). The demodulation of sample point spectra were done using a vector phase sensitive detection (PSD) to get phase resolved absorbance spectra (4).

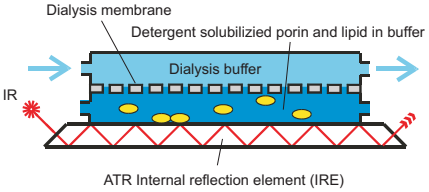

Fig. 2: Arrangement of the ATR dialysis cell for in situ FTIR measurements of porin monolayer assembly.

Results

In situ Preparation of an oriented, monomolecular Omp32/lipid layer

Dialysis: In situ monitoring of dialysis shows the progress of the adsorption process of the Omp32/lipid film onto the Ge ATR plate (Fig. 3). The time dependent behaviour of the components as shown in Fig. 4 were evaluated from time resolved difference spectra, taking the spectrum at the end of dialysis as reference. The observation of a saturation in the course of Omp32 adsorption strongly suggests that a monolayer is formed.

Calculation of surface concentrations: Quantification of porin, DMPC and DM-d67-PC in adsorbed films has been performed using the spectra measured after 17-19 h of H-D exchange. Surface concentrations were calculated from measurements with parallel and perpendicular polarized incident light, using the amide I' band for Omp32, and the symmetric stretching vibration of the methylene groups ($_s(CH_2)$ and $_s(CD_2)$) for DMPC or DM-d67-PC, respectively. Omp32/lipid 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 which showed a trimer area of 87 nm^2 (6). In addition, Omp32 exists in two 3D crystal forms (7). The one with the smaller unit cell size (CF2) exhibits a lateral arrangement leading to an area per trimer of 67 nm^2 which fits exactly our mean area. This result approve the assumption of a monomolecular film adsorption. Two to six DMPC molecules per Omp32 monomer were found.

Orientation: By means of dichroic difference spectra a strong orientation of the Omp32 molecules were found. The -barrel axis is oriented preferable perpendicular to the surface of the Ge IRE.

A more detailed analysis of the results obtained by dialyses is found in (8).

Electric field excitation

Phase resolved absorbance spectra (Fig.6) of the modulated Omp32 monofilm show D_2O components which were much stronger as found in a corresponding experiment without a porin layer. This indicates a electric field depending orientation of D_2O in the water filled pore of Omp32. Small absorption bands at 1640 cm^{-1}, 1631 cm^{-1}, 1620 cm^{-1} and a broad band around 1560 cm^{-1} were reproducible detected in the modulation spectra of Omp32. Further experiments has to be done, to decide whether this bands reflect responses of -strands of the barrel or of amino acid residues (probably of arginine, lysine and acidic amino acids), e.g by varying the pH of the buffer.

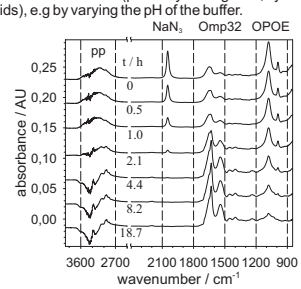

Fig. 3: Adsorption of OMP32 and deuterated DMPC to the ATR IRE of the dialysis cell. The spectra (p-polarized) show the fast decrease of the intensity of the azide band and the slower vanishing of OPOE bands. The appearance of increasing protein bands indicates unambiguously adsorption of Omp32 to the IRE. The development of the shape of the amide I is characteristic of a protein rich in anti-parallel pleated sheet structure. After 18.7h no further increase of the protein bands could be observed within one hour. There are weak, but unambiguously detectable bands resulting from the stretching vibrations (CD_2) favouring the conclusion that Omp32 porin adsorption is parallelled by the adsorption of small amounts of phospholipids.

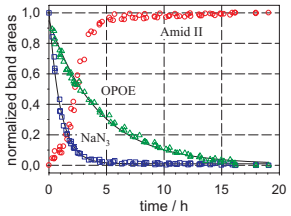

Fig. 4: Kinetics of the dialysis. Normalized integrated absorbance of NaN_3 at 2048 cm^{-1}, OPOE at 1082 cm^{-1}, and the amide II band of the protein at 1542 cm^{-1}. The decrease of NaN_3 and the detergent can be fitted by a single exponential function resulting in a time constant for the NaN_3 extraction at 35°C of 1.0±0.04 h, whereas the corresponding time constant for OPOE was found to be 4.3±0.05 h. Omp32 porin adsorption shows a different characteristic. Within the first hour of dialysis the amide II band raises slowly. Acceleration of porin adsorption starts after about 1.2 h when the OPOE concentration is reduced to 0.35 %. A saturation of Omp32 adsorption is achieved after about 4 hours and almost finished after 5 hours. The critical micelle concentration (cmc) of OPOE of 0.25 % is reached after 3 h of dialysis.

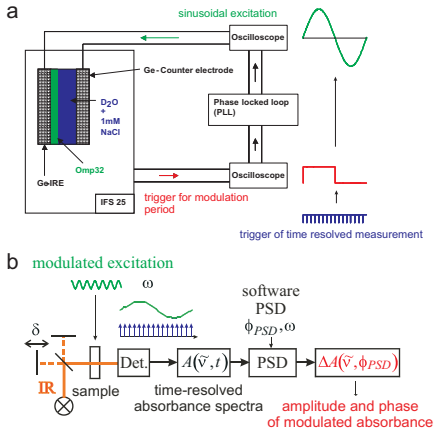

Fig. 5a,b: Setup for electric field modulation of Omp32 monolayer and phase sensitive detection (PSD) of time resolved measurements. ***a*** At the beginning of each modulation period (5 min) a trigger is produced by the spectrometer. A phase locked loop converts this trigger impulses into a sinusoidal voltage with a peak to peak amplitude of 1 V. This electric signal is used to produce the electric field excitation of the Omp32 monolayer. The electrodes are the ATR-IRE and the cell back-wall (germanium plate). ***b*** 16 time resolved spectra (sample point spectra) are sampled synchronously with modulated excitation. The demodulation of sample point spectra is done by a vector phase sensitive detection (PSD) to get phase resolved absorbance spectra (4).

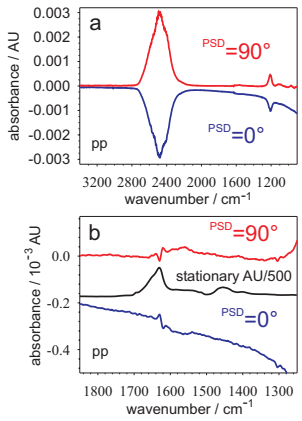

Fig. 6a,b: Phase resolved spectra of electric field modulation excitation spectroscopy of an Omp32 monolayer (p-polarized). ***a*** Strong D_2O components in the phase resolved spectra indicate a electric field depending orientation of D_2O in the water filled pore of Omp32. ***b*** Small absorption bands at 1640 cm^{-1}, 1631 cm^{-1}, 1620 cm^{-1} and a broad band around 1560 cm^{-1} were reproducible detected in the phase resolved spectra. These bands reflect only the changes in absorbance effected by the changes of the electric field during the modulated excitation. The band shape of the amid region of stationary spectra is shown in a reduced scale (1/500) to indicate the difference between general protein absorption and changes induced by the electric field. The sensitivity of modulated excitation spectroscopy is well demonstrated by the achieved signal to noise ratio.

References

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