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Crystallization and preliminary X-ray crystallographic studies of the native and chemically modified anion-selective porin from *Comamonas acidovorans*

Kornelius Zeth,[a] Volker Schnaible,[b] Michael Przybylski,[b] Wolfram Welte,[c] Kay Diederichs[c] and Harald Engelhardt[a]*
at [a]Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany, [b]Universität Konstanz, Fakultät für Chemie, PO Box 5560 M656, D-78434 Konstanz, Germany, and [c]Universität Konstanz, Fakultät für Biologie, PO Box 5560 M731, D-78434 Konstanz, Germany. E-mail: engelhar@biochem.mpg.de

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Abstract

Omp32, the strongly anion-selective porin from *Comamonas acidovorans*, has been crystallized. Two crystal forms were observed, both of which belong to space group *R*3, but exhibit different cell dimensions $a = b = 106.7$, $c = 140.6$ Å (crystal form I) and $a = b = 87.1$, $c = 135.3$ Å (crystal form II) with one trimer per asymmetric unit. The crystals diffract to 2.2 and 2.3 Å resolution, respectively. Omp32 was chemically modified by introducing negative charges through succinylation. The number and positions of the individual modifications were determined using mass spectrometry and X-ray crystallography. Chemically modified porins yielded crystals of a third form, also of space group *R*3 but with cell constants of $a = b = 109.3$ and $c = 263.2$ Å (crystal form III), showing a virtually doubled *c* axis. Crystals of form III diffract to 3.5 Å resolution.

1. Introduction

The outer membrane of Gram-negative bacteria contains pore-forming proteins, known as porins, which act as a coarse sieve to exclude large molecules but allow the passage of polar solutes such as ions, small sugars or organic acids (Nikaido, 1993). The structures of five nonspecific bacterial porins have been determined by X-ray crystallography (Weiss *et al.*, 1991; Cowan *et al.*, 1992; Kreusch *et al.*, 1994; Hirsch *et al.*, 1997). All are built on similar structural principles. They form homotrimers of 16-stranded antiparallel β-barrels with a similar shape and tilt of the β-strands. The N- and C-termini are located in the periplasmic space, except for OmpF and PhoE where they are integral parts of the β-barrel. Eight loops connect neighbouring strands at the extraplasmic surface while seven or eight short hairpin turns are found at the periplasmic surface. The typical exclusion limit for nonspecific porins was estimated to be ~600 Da (Nikaido & Vaara, 1985; Welte *et al.*, 1995) and is mainly controlled by the size of the pore constriction zone formed by loop 3 folding back into the pore. The structural conditions of functional properties concerning the passage of ions (*i.e.* ion selectivity and asymmetric conductance) through non-specific porins are largely unclear but they are believed to be correlated with the number and distribution of charged amino acids in the pore and around the pore mouth (Przybylski *et al.*, 1996).

The porin Omp32 from *Comamonas acidovorans* is interesting in its structural and functional properties which were discovered through X-ray crystallography and conductance measurements. The anion selectivity of Omp32 is high (factor of discrimination of anions over cations: 22) compared with values of structural known porins (Benz & Bauer, 1988) and its unusual asymmetric and nonlinear conductance behaviour in planar lipid membranes is remarkably different to that of other porins (Mathes & Engelhardt, 1997). Positively charged residues in the pore mouth seem to play an important role for both phenomena. In order to learn about the influence of these charged residues the porin Omp32 has been studied in its structural and functional details. Omp32 was, therefore, purified, crystallized and chemical modifications of lysine residues were performed using succinic anhydride. Matrix-assisted laser desorption ionization (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) was used to localize number and positions of chemically modified residues. X-ray structure determination of native and modified porin Omp32 combined with studies on single channel conductance will provide clues as to the function of positively charged amino acids in this anion-selective porin.

2. Materials and methods

2.1. *Purification of porin*

Omp32 was extracted from the outer membrane of a surface-protein deficient mutant of *C. acidovorans* (source: type strain DSM 39) by means of octyl-polyoxyethyelene (OPOE, Bachem) according to the procedure described in Brunen *et al.* (1991). Porin was purified by gel filtration on a Superdex-200 column (HiLoad column 16/60, Pharmacia) equilibrated with 0.6% OPOE, 10 m*M* phosphate pH 7.5, 3 m*M* NaN_3 followed by anion-exchange chromatography using Q-Sepharose fast-flow medium (HiLoad column 16/10, Pharmacia). Elution was carried out with a gradient of 0–1 *M* NaCl, 0.6% OPOE, 20 m*M* Tris, 3 m*M* NaN_3, pH 7.5. SDS–PAGE was used to determine the purity of Omp32. Samples for SDS–PAGE were prepared under denaturing conditions (samples were heated at 393 K for 15 min) and native conditions, *i.e.* $T \leq 303$ K where porins are not denatured and run as trimers (Gerbl-Rieger *et al.*, 1988). The porin samples were finally concentrated with Amicon ultrafiltration units and dialysed against crystallization buffer (10 mg ml^{-1} porin, 2% β-D-octylglucoside, 20 m*M* Tris, pH 8).

2.2. *Succinylation of porin*

The succinylation of Omp32 was carried out similar as described previously (Przybylski *et al.*, 1996). The reaction mixture was prepared by diluting a protein stock solution (10 mg ml^{-1} protein, 0.6% OPOE, 10 m*M* phosphate buffer pH 6.5) with a solution of 0.6% OPOE, 300 m*M* phosphate buffer pH 6.5 to a final protein concentration of 1 mg ml^{-1}. The reaction was started by addition of small amounts of succinic anhydride dissolved in dry DMSO (100 mg ml^{-1}) of 600 µl of protein solution under stirring. The pH of the reac-

tion mixture was maintained at 6.5 for 30 min with a pH-stat by automated addition of small amounts of a 1 *M* NaOH and a 1 *M* HCl solution. The molar ratio of reagent to lysine residue of porin was chosen to be one-, ten- and 100-fold (corresponding samples were named S1, S10, and S100, respectively). Succinylated porin was concentrated to a final concentration of 10 mg ml $^{-1}$, dialysed against crystallization buffer and directly used for dynamic light-scattering studies. Crystalization trials, MALDI- and ESI-MS.

2.3. *Dynamic light scattering*

Dynamic or quasi-elastic light scattering was performed with a DynaPro-801 instrument (Proteins Solutions, Inc). Samples of native and modified porin with 5 mg ml $^{-1}$ in 1% β-D-octylglucoside, 10 m*M* Tris pH 8 were used. All protein samples were filtered using a Whatman Anotop Plus syringe filter of 0.1 µm porosity while injecting the samples. The data returned from the instrument are: scattering amplitude, translational coefficient, particle radius, estimated molecular weight, polydispersity, temperature in the cell, counting, baseline and sum of squares. The baseline represents the completeness and fit of the regression applied during the analysis and is used to characterize the size distribution. The sum-of-squares measurement compares the fit between the experimental data and an autocorrelation function generated from the analysis results.

2.4. *Fourier transform infrared spectroscopy (FTIR)*

The secondary-structure composition of Omp32 was assessed by attenuated total reflection FTIR, using Ge crystals, and band form analysis as described previously (Engelhardt *et al.*, 1990). Porin samples were dialysed against *aqua dest.* to remove buffer salts and most of the detergent. Spectra were recorded from samples of 100 µg by adding 512 scans at a resolution of 2 cm $^{-1}$.

2.5. *Crystallization of porins, data collection and processing*

All crystals were grown at 290 K using the hanging-drop vapor-diffusion method. Hanging drops were prepared by mixing 5 µl of porin in 2% β-D-octylglucoside, 20 m*M* Tris buffer pH 8 with 5 µl of the corresponding reservoir solution. Crystals of form I and III were grown in a solution of 10 mg ml $^{-1}$ protein concentration with 1.3 to 1.4 *M* Li_2SO_4, 100 m*M* Hepes, pH 7.5 or 1.7 to 1.8 *M* $(NH_4)_2SO_4$, 100 m*M* Tris, pH 8.6 in the reservoir. Crystals appeared after a few days. Crystals of form II appeared after several months from a 10 mg ml $^{-1}$ protein solution with a reservoir of 1.3 *M* $(NH_4)_2SO_4$, 100 m*M* Tris, pH 8.6. The data sets of crystal forms I and II were taken on MAR IP using synchrotron radiation at the Max-Planck beamline BW6 (DESY, Hamburg). X-ray diffraction data of crystal form III and of the heavy-atom derivatives were collected by means of a rotating-anode source (STOE, Darmstadt, Germany) and a STOE image-plate detector system. Data reduction and determination of space groups was carried out using the programs *XDS* (Kabsch, 1988) and *DENZO* (Otwinowski, 1993).

3. Results and discussion

Omp32 is a strongly anion-selective porin (Mathes & Engelhardt, 1997) similar to Omp34, the porin of the related species *Acidovorax delafieldii* (Brunen & Engelhardt, 1993, 1995). According to function the sequence of Omp32 exhibits a surplus of positivity charged amino acids (Gerbl-Rieger *et al.*, 1992). In order to determine the importance of these charges for the functional properties, we have covalently modified amino groups by succinylation, *i.e.* mainly ε-amino groups of Lys. The N-terminus of Omp32 carries a pyroglutamate and is, therefore, protected (Gerbl-Rieger *et al.*, 1991). Every bound succinate group (M_r = 100) removes one positive charge and introduces a negative one, changing the ion selectivity properties of the porin. Non-denaturing SDS–electrophoresis of S1 showed that succinylation did not lead to dissociation of the porin trimer and, therefore, its quarterly structure. The FTIR-spectrum of porin sample S100 was very similar to that of native porin except for vibration signals due to bound succinyl residues (Fig. 1). It revealed a high content of β-sheet structure (~60%; Engelhardt *et al.*, 1990) and consequently the conservation of the secondary structure.

All molecular masses of modified porins were determined by MALDI-MS and for the native porin and S1 additionally with ESI-MS. The molecular mass for the native porin of 34 776 Da and 34 820 Da estimated with ESI-MS is in close agreement with the sequence-drived mass of 34 796 Da. The masses of porins S1, S10 and S100 (Fig. 2) correspond to an average degree of succinylation of 3.5, 6.5 and 15.3 residues while 17 lysines are present in the sequence. The ESI-MS spectrum of S1 could clearly resolve three different masses of 35 020, 35 120 and 35 220 Da corresponding to a mixture of two-, three and fourfold modified porin showing the selective modification of lysine residues under the reaction conditions chosen. The structural positions of the particular charges being modified might be of significance for their contribution to function, *i.e.* the effects on the pore cross-section and on the

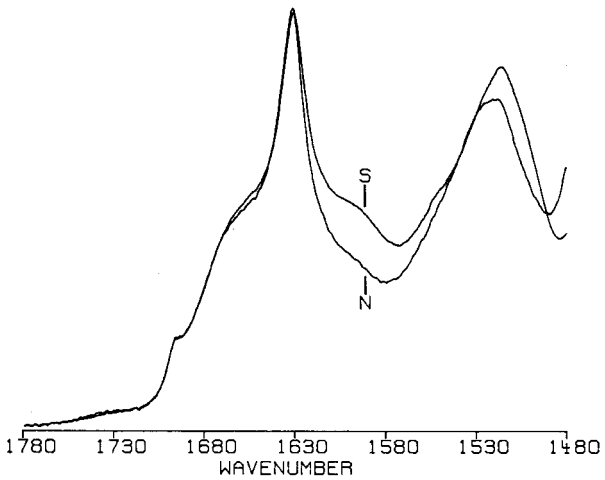

Fig. 1. FTIR spectra of the native (N) and succinylated S100 (S) porins of *C. acidovorans*. Only the amide I and II regions are shown, centred at 1630 and 1520 cm $^{-1}$, respectively. The amide I band is particularly indicative for the secondary-structure composition of proteins. The sharp component at 1630 cm $^{-1}$ is related to β-sheets and the shoulder at 1695 cm $^{-1}$ to antiparallel β-sheet and β-turn components. Note that succinylation had no effect on the secondary-structure composition. The differences between the spectra originate from bound succinyl residues (at 1590 and 1510 cm $^{-1}$) and can be addressed to asymmetric stretching vibrations of carboxylate groups and deformation vibrations of secondary amides.

Table 1. *Dynamic light-scattering measurements of native and succinylated Omp32*

Sample	Hydrodynamic radius (nm)	Polydispersity (nm)	Estimated M_r (kDa)	Baseline values
Omp32	4.6	1.05	120	1.000
S100	4.7	1.14	122	1.001

surface charges with respect to porin selectivity and diffusion of ions (Przybylski *et al.*, 1996; Brunen & Engelhardt, 1993, 1995).

Dynamic light-scattering measurements indicate homogeneous distribution of molecules in all solutions of native and chemically modified porins tested and returned an estimated molecular mass of porin–detergent micelles of about 120 kDa which is in agreement with the trimeric structure (Table 1). The baseline value given for the samples indicate that the actual distribution of molecules has been fully resolved using the theoretical size-distribution assumption. A coarse crystallization screening matrix (Jancarik & Kim, 1991) was primarily applied to native Omp32 solubilized in a number of detergents giving crystals only for β-D-octylglucoside. Detergents such as LDAO, C_8E_4 and dodecyl-maltoside did not lead to crystallization. Three precipitants (lithium sulfate, ammonium sulfate, PEG 4000) were found to yield small crystals and the refinement of pH and other parameters led to well diffracting crystals under two conditions.

Crystals of type I grew within 2–3 d with dimensions up to 300 × 300 × 200 μm. The crystals exclusively grew in phases which had been developed within the original solution (Fig. 3). The size of the crystals was mainly controlled by the size of the protein enriched phase around the crystals. The crystals belong to space group *R*3 and have cell dimensions of $a = b = 106.7$ and $c = 140.6$ Å. There is one porin monomer in the asymmetric unit. A data set to 2.2 Å resolution of 92.7% completeness ($R_{sym} = 13\%$) was collected, containing 18 160 unique reflections. Crystals of type II grew from precipitated protein after 4–6 months to dimensions of 250 × 250 × 250 μm. The crystals belong to the same space group *R*3 but exhibit smaller cell constants of $a = b = 87.1$ and $c = 135.3$ Å. Diffraction data were collected to 2.3 Å resolution and 96.2% completeness ($R_{sym} = 10\%$) with 16 453 unique reflections. The difference in crystal packing of type I and type II crystals is remarkably high and suggests clearly different protein contacts. Crystals of all succinylated porins (S1 and S100) grew to maximum dimensions of 150 × 150 × 150 μm. These crystals are again of space group *R*3 and possess an almost doubled cell axis in the *c* direction ($c = 263.2$ Å) but show only slightly larger cell axes in the other directions ($a = b = 109.3$ Å compared with crystal form I. All succinylated crystals diffract to about 3.5 Å resolution. Changes in the charge profile of the porin and in the structure of individual lysine residues located on the surface of the porin lead to large differences in the crystal packing and new association of porin trimers in the crystal. Whether heterogeneity in succinylation of individual molecules exerted some influence on the resolution remains to be shown.

The structure solution of native Omp32 using the method of molecular replacement with all of the known porin structures as search models was not successful analogous to the attempt of the structure solution of the *Rhodopseudomonas blastica* porin using the same technique (Kreusch *et al.*, 1994). This

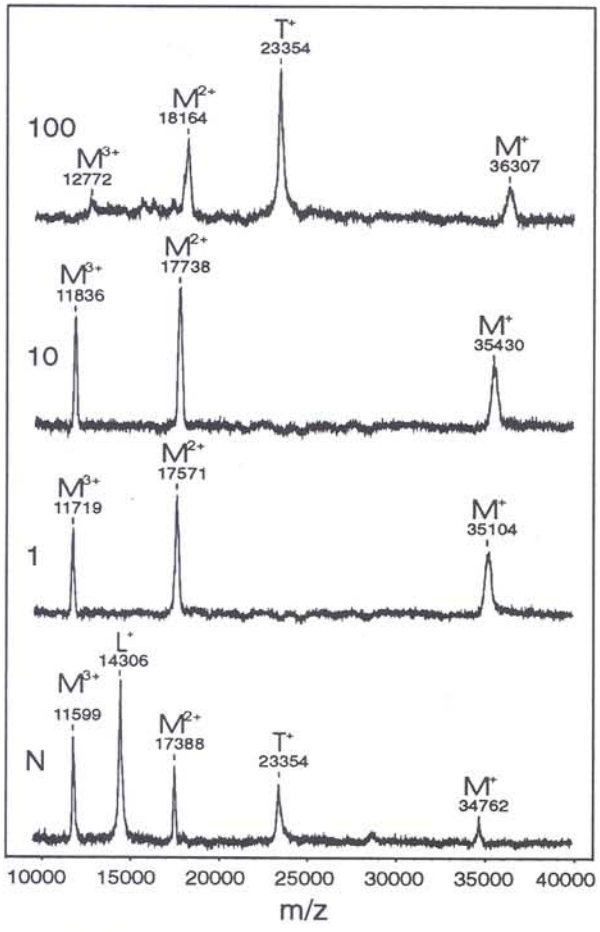

Fig. 2. MALDI-MS analysis of the native porin (N) and of succinylated porins S1 (1), S10 (10), and S100 (100). Mass calibration was performed by using the precise average isotope signals of the singly charged molecular ions trypsin (T^+) and lysozyme (L^+) as internal standards. The masses of the native and modified porins were calculated from the calibrated M^{2+} molecular porin peaks.

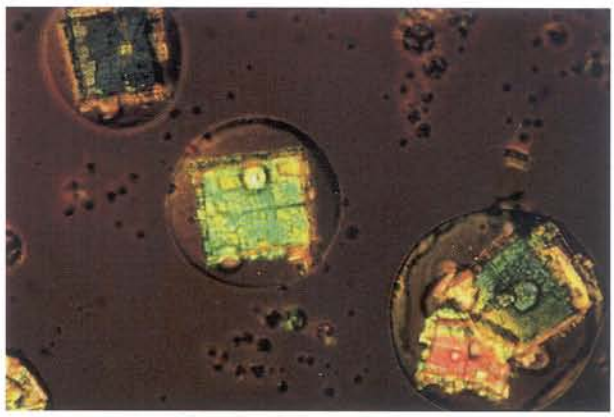

Fig. 3. Crystals of native porin Omp32 (crystal form I) growing within 2–3 d in presence of 2% β-D-octylglucoside. The crystals exclusively occur in droplets containing a protein-enriched phase due to spontaneous phase separation. The dimensions of the crystals are determined by the size of the corresponding phase. Size of the crystal in the centre 200 × 200 × 150 μm.

indicates that significant differences can be expected between the structure of Omp32 and the other non-specific porins. In order to solve the structure through multiple isomorphous replacement the search for heavy-atom derivatives was started with crystals of type I. The first heavy-atom derivative of very good quality was produced by soaking porin crystals with 5 m*M* K_2PtCl_4 for 24 h giving high phasing power up to 4.0 Å. A second derivative of lower quality could be found by soaking crystals with 1 m*M* $La(NO_3)_3$ for 48 h. The determination of the porin structure is in progress.

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