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Isolation, functional characterization and crystallization of Aq_1259, an outer membrane protein with porin features, from *Aquifex aeolicus*

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ABSTRACT

The "hypothetical protein" Aq_1259 was identified by mass spectrometry and purified from native membranes of *Aquifex aeolicus*. It is a 49.4 kDa protein, highly homologous (>52% identity) to several conserved hypothetical proteins from other bacteria. However, none of these proteins has been characterized using biochemical or electrophysiological techniques. Based on the sequence and circular dichroism spectroscopy, the structure of Aq_1259 is predicted to be a β-barrel with 16 β-strands. The strands with loops and turns are distributed evenly through the entire sequence. The function of Aq_1259 was analyzed after incorporation into a lipid bilayer. Electrophysiological measurements revealed a pore that has a basic stationary conductance of 0.48 ± 0.038 nS in a buffer with 0.5 M NaH_2PO_4 at pH 6.5 and 0.2 ± 0.015 nS in a buffer with 0.5 M NaCl at pH 6.5. Superimposed on this is a fluctuating conductance of similar amplitude. Aq_1259 could be crystallized. The crystals diffract to a resolution of 3.4 Å and belong to space group *I*222 with cell dimensions of a = 138.3 Å, b = 144.6 Å, c = 151.8 Å.

1. Introduction

As DNA sequencing techniques are developing, a huge amount of genomic information has been obtained by large-scale genome sequencing projects (http://www.genomesonline.org/gold_statistics.htm). Gene products are annotated based on sequence homology to previously characterized proteins from other organisms [1]. However, in any newly sequenced genome, 30% to 50% of the encoded proteins have either no detectable homology or show too little sequence homology (less than 30% identity) to known proteins for a reliable function assignment [2]. These potential gene products are known as "hypothetical proteins" or "putative proteins". Some of them are predicted to be membrane proteins [3].

Gram-negative bacteria are surrounded by two membranes: the inner (cytoplasmic) membrane and the outer membrane. The space between the two membranes is called the periplasmic space. Solvents and nutrients need to permeate into the periplasmic space through pore-forming outer membrane proteins called porins. A low sequence homology and an inconsistent functional characterization make it difficult to compare and classify porins from different species. In the transport DB database (http://www.membranetransport.org), outer membrane proteins are classified in different families according to the substrate specificity based on biochemical analyses and phylogenetic data [4]. Three classes of porins are described: general non-specific porins, substrate specific porins and porins associated with active transporters [5]. The best studied porins are the general non-specific porins, OmpF, OmpC and PhoE from *Escherichia (E.) coli*. The substrate specific porins that have been characterized are the nucleotide transporter TsX [6,7], the maltose channel LamB [8,9] and a phosphate specific porin, OprO [10,11]. Active transporters, such as FhuA, bind a substrate and can transport it against a concentration gradient [12,13]. The energy required for the transport is supplied by the inner membrane protein TonB.

The properties of the general porins have been studied for many years. They are large channels without substrate binding sites [14–16] and are water-filled which facilitates the diffusion of small polar substrates (<600 Da) across the membrane [17]. Their crystal structures indicate that they form a trimer of β-barrels. Recent studies of the conductance of substrates of *E. coli* OmpF indicate that transport across the outer membrane may be more complicated than thought previously [18,19].

Most studies of porins have been carried out on porins from proteobacteria like *E. coli* and *Salmonella typhimurium* while neglecting porins from other bacteria, such as thermophiles. These bacteria live in an extreme environment and expected to have specific devices for transport across the outer membrane. The studies of porins from *Thermotoga maritima* and *Thermus thermophilus* have yielded very little information so far [20].

Based on the sequence information, six outer membrane proteins are annotated for *Aquifex (A.) aeolicus* [21]. In the Transport DB database, several hypothetical proteins are predicted to be outer membrane

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proteins. Aq_1862 is the only porin that has been identified in *A. aeolicus*, also the only one investigated in detail from a hyperthermophilic bacterium [22].

The "hypothetical protein" Aq_1259 was identified and purified from the native membranes of *A. aeolicus*. The amino acid sequence shows no significant homology to other proteins. To investigate its function, bioinformatics, electrophysiological analyses and crystallization trials were performed. The results demonstrate that the protein has pore-forming activity and the general structural properties of porins.

2. Materials and methods

2.1. Purification

A. aeolicus cells were obtained from Archaeenzentrum Regensburg, Germany. Membrane isolation and solubilization were performed as described previously [23]. Aq_1259 was purified from *A. aeolicus* membrane fraction solubilized by 3% (w/v) dodecyl-β-D-maltoside (DDM). The solubilized membrane proteins were loaded onto a Mono Q HR 10/10 column (Amersham Biosciences) pre-equilibrated with 20 mM Tris–HCl, pH 7.4, 0.05% (w/v) sodium azide, and 0.05% (w/v) DDM and eluted with a linear gradient of 0.0–1.0 M NaCl. The detergent of Aq_1259 containing fractions (judged by SDS-PAGE) was exchanged by octyltetraoxyethylene (C_8E_4) for purification using a Mono Q HR 10/10 column. Aq_1259 containing fractions were collected and loaded onto a Superdex 200 column (Amersham Biosciences). The purified protein was stable in the presence of C_8E_4 at 4 °C.

2.2. Peptide mass fingerprinting (PMF)

Identification of SDS-PAGE separated proteins was performed on reduced and trypsin-digested samples prepared by standard mass spectrometry protocols (Proteoextract Digestion kit, CalBiochem). The proteolytic digests were loaded onto nano-HPLC (Proxeon easy-nLC) reverse phase columns (trapping column: particle size 5 μm, C18, L = 20 mm; analytical column: particle size 5 μm, C18, L = 10 cm or 15 cm; NanoSeparations, Nieuwkoop, The Netherlands), and eluted using gradients of water (0.1% formic acid, buffer A) and acetonitrile (0.1% formic acid, buffer B). Typically, gradients were ramped from 5% to 65% B in 25 min at flow rates of 300 nl/min (extended gradients: 5% to 65% B in 60 min). Peptides eluting from the column were ionized online using a Bruker Apollo ESI-source with a nanoSprayer emitter and analyzed in a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-Q-II and Bruker maXis). Mass spectra were acquired over the mass range 50–2200 m/z, and sequence information was acquired by computer-controlled, data-dependent automated switching to MS/MS using collision energies based on mass and charge state of the candidate ions.

The data sets were processed using a standard proteomics script with the software Bruker Data Analysis 4.0 Service Pack 1 Build 253 and exported as Mascot generic files. Spectra were internally recalibrated using autoproteolytic trypsin fragments when applicable.

Proteins were identified by matching the derived mass lists against the NCBInr database (downloaded from http://www.ncbi.nlm.nih.gov/ on 19/08/2009) on a local Mascot server (Version 2.2.2, Matrix Science, UK). In general, a mass tolerance ± 0.05 Da for parent ion and fragment spectra, two missed cleavages, oxidation of Met and fixed modification of carbamidomethyl cysteine were selected as matching parameters in the search program.

These data were analyzed and validated using the BioTools 3.1 software package (build 2.22, Bruker Daltonics).

2.3. Sequence comparison

A BlastP search was performed using the NCBI blast server with the standard setting [1]. For signal peptide prediction the program SignalP [24] was used. The transmembrane β-barrel prediction was carried out by the program PRED-TMBB [25] and the fold compared by the programs HH-PRED [26] and phyre^2 [27].

2.4. Circular dichroism spectroscopy

The secondary structure of Aq_1259 was monitored by performing a far-UV CD spectroscopic analysis [28]. Far-UV CD measurements were performed on a Jasco 720 CD spectropolarimeter (Jasco, Easton, MD) at temperatures of 20–22 °C, using a 0.1 cm high transparency quartz cuvette. The measurement was performed with a purified sample (0.4 mg/ml). Ten scans were accumulated for each spectrum with a response time of 1 s, a bandwidth of 1.0 nm and a scan speed of 200 nm/min in the range of 200–250 nm.

2.5. Lipid bilayer experiments

Lipid bilayer experiments were performed as described before [22]. In brief: painted lipid bilayers were preformed across an aperture (0.1 mm diameter) separating two half-cells (volume 1.4 ml) of a Teflon cuvette. The aperture was pretreated with 0.5% (w/v) diphytanoyl phosphatidylcholine in hexane, and lipid bilayers were formed from 1.5% (w/v) diphytanoyl phosphatidylcholine (Avanti Polar lipids, Birmingham, UK) in n-decane. The cuvette was connected to the measuring circuit with Ag/AgCl electrodes via salt bridges (3 M KCl, 5% Agar). One compartment was connected to a variable voltage source and the other one was connected to a current to voltage converter (Standford Research Systems, Sunnyvale, Ca; or custom made: amplification 10^9 A/V, bandwidth dc to 1 kHz). Signals were digitized (MiniDigi 1A, Axon Instruments, Foster City, CA; sample-rate 1000 Hz) and recorded on a PC. For recording and analysis of the data, the pCLAMP 9 program package (Axon Instruments) was used.

The buffers were 5 mM Tris–HCl pH 6.5 with 0.5 M NaCl or NaH_2PO_4. The pH of buffers was adjusted after adding the salts. The final concentration of Na^+ in the buffer with NaH_2PO_4 is around 0.63 M, in the buffer with NaCl it is around 0.51 M. The experiments were carried out between 20 and 22 °C. The protein concentration was 0.1 μg/ml in 20 mM Tris–HCl (pH 6.5) containing 0.5% C_8E_4. The protein concentration was determined by the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as protein standard. The lipid was painted over the hole to form the membrane and, subsequently, the protein was added to the compartment connected to the amplifier, (cis-side) before forming the membrane. Under these conditions membranes with one conducting unit could be obtained (see below and Ref. [22])

2.6. Crystallization and data collection

The protein was concentrated to a concentration of 4 mg/ml in a buffer of 20 mM Tris–HCl at pH 7.4, 150 mM NaCl and 0.5% C_8E_4. Standard crystallization screening was carried out using crystallization robots (Honeybee, Cartesian). Crystallization screens were purchased from Sigma, Jena Bioscience and Qiagen. Three hundred nanoliters of protein solution was mixed with crystallization buffer in a ratio of 1:1, sealed and kept at 18 °C. Crystallization was done by vapor diffusion in sitting drops and hanging drops. All datasets were collected at the Swiss Light Source, beamline X10SA (Villigen, Switzerland).

3. Results and Discussion

3.1. Identification and purification of Aq_1259

Aq_1259, annotated as "hypothetical protein" (Swiss-Prot #O67300), was purified from DDM solubilized membranes of *A. aeolicus*. The purification steps involved anion exchange chromatography, gel filtration, a