Pore-Forming Activity of Outer Membrane Extracts from the Unicellular Cyanobacterium *Synechocystis* sp. PCC 6714

Uwe J. Jürgens

Institut für Biologie II, Mikrobiologie, der Albert-Ludwigs-Universität, Schänzlestraße 1, D-7800 Freiburg i. Br., Bundesrepublik Deutschland

Roland Benz

Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, Bundesrepublik Deutschland

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Cell walls of the unicellular cyanobacterium *Synechocystis* sp. PCC 6714, isolated from cell homogenates, were found to be unusually resistant against extraction with various detergents, organic solvents, chaotropic agents, and proteases. The major outer membrane proteins (M_r 67,000; 61,000; 94,000) were solubilized by differential SDS-extraction and purified by preparative SDS-PAGE. The extracted proteins, reconstituted into lipid bilayer membranes, formed two types of pores with single-channel conductances of 2.2 nS (pore diameter of 1.4 nm) and 0.3 nS (pore diameter not determined), respectively. Carotenoids and lipopolysaccharide were found to be associated with the extracted major proteins.

Introduction

Cyanobacteria have an outer membrane which consists of lipopolysaccharide, proteins, lipids, and carotenoids [1–5]. Phosphatidylglycerol, sulfolipid, phosphatidylcholine, and unidentified lipids [3, 4] are the lipids found in the outer membrane of the unicellular *Synechocystis* sp. PCC 6714. The carotenoid spectrum of isolated outer membrane fractions comprises myxoxanthophyll, related carotenoid-glycosides, zeaxanthin, echinenone, and β-carotene. The polypeptide patterns of the outer membrane of *Synechocystis* sp. PCC 6714 are dominated by only a few major proteins. Thus, the outer membrane of cyanobacteria might have structural and functional similarities in common with the outer membrane of gram-negative bacteria, although the peptidoglycan of cyanobacteria has properties of peptidoglycan from gram-positive bacteria [6, 7].

Pore-forming proteins ("porins") allow the passage of small hydrophilic solutes across the outer membrane of gram-negative bacteria [8–10]. They are peptidoglycan-associated and form heat-modifiable oligomers with an average molecular weight between 30,000 and 50,000 of the monomer. Although also being peptidoglycan-associated, the major outer membrane proteins of cyanobacteria are not heat-modifiable and have an apparent molecular weight above 50,000. Little is known about their function. A protein (M_r 40,000 to 80,000) with pore-forming activity in lipid bilayers and having an apparent molecular weight of 40,000 to 80,000 in SDS-PAGE has been described for the outer membrane of the filamentous cyanobacterium *Anabaena variabilis* [11]. A pore diameter of 1.6 nm was estimated from single-channel experiments. This corresponds to an exclusion limit of around M_r 2000. In addition, a small pore (average conductance of 0.3 nS) was observed in the same strain. In contrast to porins from enteric bacteria, the pores of *Anabaena variabilis* seem to be voltage-dependent, similar to pore-forming proteins from the outer membrane of chloroplasts and mitochondria [12, 13]. In this study, we demonstrate pore-forming activity of outer membrane extracts from the unicellular *Synechocystis* sp. PCC 6714. The extracts contain major outer membrane proteins in close association with lipopolysaccharide and carotenoids.

Abbreviations: EDTA, ethylene diamine tetraacetate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Reprint requests to Dr. U. J. Jürgens.

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Materials and Methods

Strain and culture conditions

Synechocystis sp. PCC 6714 was obtained from the Pasteur culture collection (PCC), Paris. Mass cul-

tures of the strain were grown photoautotrophically in a Biostat E fermentor (10 l, Braun, Melsungen, F.R.G.) using BG-11 medium, pH 7.5 [14] at 25 °C and 5000 lx under continuous aereation by a stream of air/CO_2 (99:1, v/v, at 250 l h^{-1}). Cells were harvested after 18 d, washed once with 20 mM Tris/HCl buffer, pH 8.0, and stored at −20 °C until use.

Isolation of cell walls and outer membranes

Cell walls and outer membranes were separated from cell homogenates after mechanical disruption of cells by differential centrifugation and were purified from thylakoid- and cytoplasmic membranes by sucrose density centrifugation, Triton X-100 extraction [15] followed by lysozyme digestion were as previously described [4, 16].

Purification of outer membrane proteins

Outer membrane proteins were solubilized from cell walls by differential SDS-extraction (2% SDS, 10% glycerol, 20 mM Tris/HCl buffer, pH 8.0; 30 min) at different temperatures ranging from 30 °C to 90 °C. After SDS-extraction the cell wall suspensions were centrifuged (48,000 × *g*, 15 °C, 30 min) and the supernatants were dialyzed against distilled water at room temperature before lyophilization. The supernatants (SDS-extracts of cell walls at 70 °C or higher temperature) containing major outer membrane proteins were subjected to SDS-PAGE and the protein bands were visualized with 0.2 M KCl. Major polypeptides were removed from the polyacrylamide gel by electro-elution in small dialysis tubes filled with 25 mM Tris/HCl buffer, pH 8.3, 0.19 M glycine, 0.1% (w/v) SDS at room temperature for 12 h with a constant current of 60 mV. After electro-elution the gel slices were removed by centrifugation (5000 × *g*, 15 min) and the supernatants dialyzed against distilled water.

Porin activity measurements

Before and after separation by preparative SDS-PAGE, outer membrane proteins from SDS-extracted cell walls were solubilized in 2% (w/v) Triton X-100 and reconstituted in black lipid bilayer membranes as described previously [17, 18]. The experiments were performed in 1 M KCl, pH 6.0, at 25 °C. Porin was added from a concentrated protein solution (1 mg/ml) either immediately before membrane formation or after the membrane had turned black. The single-channel conductance of the pores was measured after application of a fixed membrane potential with a pair of calomel electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane. The current through the membrane was boosted by a current amplifier, monitored on a storage oscilloscope, and recorded on a strip chart or tape recorder.

Preparative SDS-PAGE

SDS-PAGE was performed as described previously [7]. For preparative purposes, protein bands were visualized with 0.2 M KCl, removed from SDS-gels (2 mm i.d.) by electro-elution (60 mA, 12 h) in 25 mM Tris/HCl buffer, pH 8.3, containing 0.1% SDS and 0.19 M glycine, and dialyzed against distilled water.

Results

Solubilization of major polypeptides from cell walls

SDS (2%, w/v) removed the major polypeptides from the cell walls of *Synechocystis* sp. PCC 6714 at temperatures above 70 °C (see below). Increasing the detergent concentration (up to 10%, w/v) had no significant effect on the solubilization temperature of the proteins. Detergents such as Triton X-100, N-lauroyl sarkosin, Brij-35, sodium deoxycholate, and β-*n*-octylglucoside (all: 2%, w/v, 37 °C, 30 min) did not solubilize the major outer membrane proteins, even in the presence of salts (0.15 to 0.5 molar LiCl, NaCl, KCl, CsCl) or chelating agents (10 mM to 100 mM EDTA). Organic solvents such as benzene, methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, 1-dodecanol, acetonitrile, trichloromethane, ethyl acetate, formamide, 1,4-dioxane, triethylamine, dimethylsulfoxide, or hexamethyl phosphoric acid triamide [19] did not solubilize the major proteins from cell walls except phenol (91%, w/v, 20 °C, 30 min), which solubilized a major part of the outer membrane proteins. Even strong chaotropic agents such as guanidinium hydrochloride (3 molar), guanidinium isothiocyanate (3 molar), and urea (6 molar) failed to disintegrate the outer membrane and, thus, a release of major polypeptides from cell walls was not achieved by such treatments.

Isolation and function of major outer membrane proteins

Outer membrane extracts obtained by differential SDS-extraction (temperature range from 70 °C to

90 °C) of Triton-insoluble cell walls contained three major polypeptides with M_r of 61,000, 67,000, and 94,000 along with few minor proteins (Fig. 1). The relatively high temperature which was required for their release from the outer membrane indicated strong interactions with the peptidoglycan of *Synechocystis* sp. PCC 6714. The three major proteins (67,000, 61,000, and 94,000) were not modifiable by heat, 2-mercaptoethanol, EDTA, and salts. The SDS-extracted proteins (lane F in Fig. 1) were reconstituted into lipid bilayer membranes. Two different types of pores were found in single channel conductance experiments (see the conductance fluctuations in Fig. 2): a small pore, and a large pore with average single-channel conductances of 0.3 nS and 2.2 nS, respectively (see Fig. 3 for a histogram of conductance fluctuations in 1 M KCl). A pore diameter of about 1.4 nm was calculated for the large pore, assuming that the pore is a water-filled holow cylinder [9]. The diameter of the small pore could not be estimated, because this pore is possibly a specific porin and a similar approach as used above for the large pore cannot be made for a specific porin [20]. It is also evident from Fig. 2 that these pores switched off and did not have the usual long lifetime of enterobacterial porin pores [20]. Furthermore, we observed

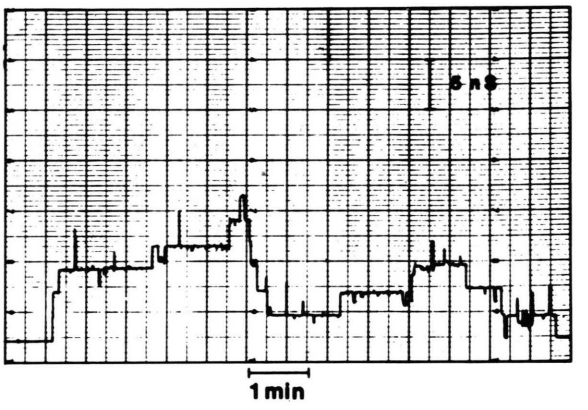

Fig. 2. Single-channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membrane after the addition of outer membrane proteins (M_r 61,000 and 67,000, solubilized from cell walls at 70 °C) of *Synechocystis* sp. PCC 6714. The aqueous phase contained 1 M KCl and 25 ng/ml protein. The membrane potential applied was 10 mV; T = 25 °C.

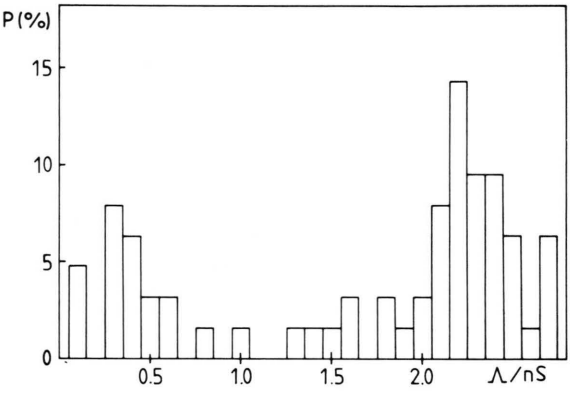

Fig. 3. Histogram of the average conductance steps measured after reconstitution of the major outer membrane proteins (M_r 61,000 and 67,000, solubilized from cell walls at 70 °C) into black lipid bilayer membranes.

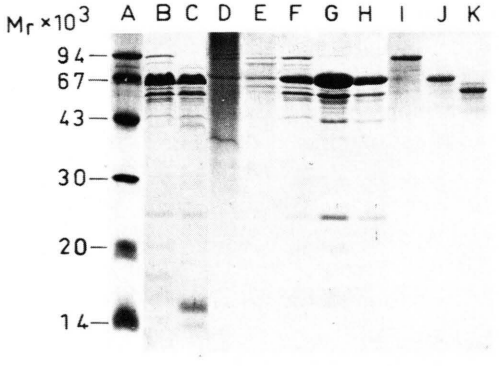

Fig. 1. Polypeptide patterns of cell walls from *Synechocystis* sp. PCC 6714 (untreated, lane B; pronase-digested, lane C), SDS-extracts of cell walls (30 °C, lane D; 50 °C, lane E; 70 °C, lane F; 80 °C, lane G; 90 °C lane H), and isolated major outer membrane proteins M_r 94,000, lane I; M_r 67,000, lane J; M_r 61,000, lane K. Standard proteins, lane A.

that the on-steps were sometimes larger than the off-steps.

Further purification of the major outer membrane proteins was achieved by preparative SDS-PAGE, followed by electro-elution and dialysis (lanes I, J, K in Fig. 1). However, single channel conductance experiments showed that the pore-forming activities were lost after electro-elution (current was 60 mA for 12 h).

Association of major proteins with outer membrane constituents

Lipopolysaccharide was present in the SDS-extracts as indicated by the presence of GlcN, 3-hydroxy fatty acids (3-OH-14:0, 3-OH-16:0), and characteristic neutral sugars [4]. In addition, carotenoids [4] were found in SDS-extracts (temperature range of extraction from 30 °C to 70 °C). They seemed to form complexes with the major outer membrane proteins (M_r 67,000 and 61,000). This became obvious after separation of the SDS-extracted proteins by sucrose density centrifugation in the presence of SDS followed by cation exchange chromatography on DEAE-Sephacel in the presence of Triton X-100. In both cases, carotenoids were found to be associated with the polypeptides (M_r 67,000 and 61,000) (Fig. 4).

Protease resistance of major outer membrane proteins

Triton-insoluble cell walls were digested with pronase in order to detect proteins localized in the outer leaflet of the outer membrane. The M_r 94,000 protein was digested by pronase. This indicated that this protein was exposed to the surface of the cell. Other proteins (including the M_r 67,000 and 61,000 polypeptides) were not accessible to the proteases. On the other hand, the major outer membrane proteins (M_r 67,000 and 61,000) were partially digested with the proteases, when peptidoglycan-protein complexes (obtained by differential SDS-extraction of Triton-insoluble cell walls) were used for the digestion.

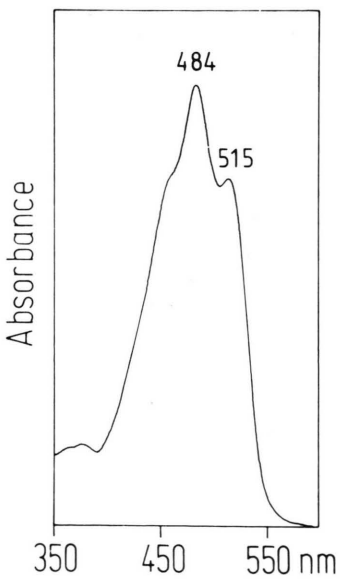

Fig. 4. Absorption spectrum of carotenoids associated with the two major outer membrane proteins (M_r 67,000 and 61,000) of *Synechocystis* sp. PCC 6714 after separation of the SDS-extract of cell walls (70 °C, 30 min) on DEAE-Sephacel.

Discussion

The major outer membrane proteins of *Synechocystis* sp. PCC 6714 were solubilized only by application of drastic extraction methods, such as sodium dodecyl sulfate treatment at temperatures above 70 °C or phenol extraction at room temperature. This finding is not exclusively due to strong interactions of the major polypeptides with the peptidoglycan as reported previously [4, 16], since isolated outer membranes of *Synechocystis* sp. PCC 6714 showed a comparable resistance against detergent treatment. Thus, ionic interactions of proteins with the peptidoglycan layer and hydrophobic protein-protein interactions or interactions of proteins with other outer membrane constituents (lipopolysaccharide, lipids, carotenoids) might contribute to the high stability of the cell walls. Similar observations were made with cell walls of *Synechococcus* sp. strains [2]. A partial solubilization of the major outer membrane proteins by 10 mM EDTA as shown with *Synechococcus* sp. [2, 21] or by 0.5 M NaCl or in combination with detergents [22] was not observed with *Synechocystis* sp. PCC 6714 cell walls. However, carotenoids and lipopolysaccharide were partially removed by EDTA treatment. In addition, the outer membrane was still visible on ultrathin sections (without any significant difference to untreated cell walls).

The cell walls of *Synechocystis* sp. PCC 6714 showed a similar resistance against pronase as reported for *Synechococcus* sp. [2]. Whereas the major outer membrane proteins (M_r 67,000 and 61,000) were not accessible to proteases in the intact outer membrane, the protein with M_r 94,000 was accessible indicating a peripheral location of the latter one.

Two different pore-forming activities were found in SDS-extracts of the outer membrane of *Synechocystis* sp. PCC 6714 after reconstitution of the solubilized proteins into black lipid bilayer membranes: a small pore, and a larger pore (average conduct-

ances of 0.3 nS and 2.2 nS, respectively). The size of the large pore (2.2 nS) with an approximate diameter of 1.4 nm was comparable to that of *Anabaena variabilis* [11], but was significantly smaller than the size of pores of plant chloroplasts [13] or mitochondria [12]. A small pore (0.3 nS), comparable to that of *Synechocystis* sp. PCC 6714 was also observed in comparable fractions of *Anabaena variabilis* (R. Benz, unpublished results). The size of this pore could not be determined (see Results section). For the cyanobacteria as phototrophic prokaryotes it seems likely that this pore might be involved in salt uptake from the growth medium.

Different purification methods were tried in order to separate the major outer membrane proteins (M_r 67,000 and 61,000) on the basis of molecular size, electric charge, and hydrophobicity. Successful separation was achieved only by preparative SDS-PAGE followed by electro-elution and dialysis. However, the porin activity was lost during this purification step. It might be possible that the pore-forming activity is maintained only when the protein-protein interactions of the major polypeptides (M_r 67,000 and 61,000) are intact. Although it has been shown for enterobacterial porins that lipopolysaccharides are not essential for pore-forming [23], we cannot exclude that the unusual switching-off of the cyanobacterial pores may be caused by loss of these molecules. The situation is further complicated by several observations (data not shown), which led to the assumption that the major outer membrane proteins of *Synechocystis* sp. PCC 6714 form complexes with carotenoids: first, a complex consisting of the major polypeptides (M_r 67,000 and 61,000) and carotenoids was isolated by ion exchange chromatography on DEAE-Sephacel under non-denaturing conditions. Second, a single orange band was obtained after sucrose density centrifugation (in the presence of SDS) of an SDS-extract of the outer membrane. The extract contained the major outer membrane proteins and carotenoids. The carotenoids were not covalently bound. They dissociated from the polypeptides during electrophoresis, and the outer membrane proteins isolated by preparative SDS-PAGE were found to be free of carotenoids. A carotenoid-binding protein was also found in the cytoplasmic membrane of *Synechocystis* sp. PCC 6714 [24]. Other carotenoid-protein complexes, loosely bound to the thylakoids, have been isolated from *Spirulina maxima. Aphanizomenon flos-aquae,* and *Microcystis aeruginosa* [25].

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