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Small angle X-ray scattering and transmission electron microscopy study of the *Lactobacillus brevis* S-layer protein

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Abstract. The structure of self-assembly domain containing recombinant truncation mutants of *Lactobacillus brevis* surface layer protein SlpA in aqueous solution was studied using small-angle X-ray scattering and transmission electron microscopy. The proteins were found out to interact with each other forming stable globular oligomers of about 10 monomers. The maximum diameter of the oligomers varied between 75 Å and 435 Å.

1. Introduction

Surface (S-) layer is the outermost layer of cell envelope that is present in most bacteria and in all Archaea. It consists of identical proteins that are attached to each other and to the underlying cell wall, forming a two-dimensional crystalline surface. In Gram-positive bacteria, S-layer attaches to the underlying peptidoglycan layer, in Gram-negative bacteria, S-layer attaches to the outer membrane in the cell wall, and in Archaea, S-layer attaches to the underlying plasma membrane, forming the only cell wall component in addition to the membrane. The bacterial surface (S) layer consists of proteins consisting usually of two structural domains. One domain is involved in the attachment of the S-layer subunit to the underlying cell wall and the other is involved in the S-layer self assembly into two-dimensional crystal structure [1, 2]. The structures and functions of most S-layer proteins and the mechanisms leading to the S-layer protein self assembly are poorly understood.

Two-dimensional crystal structures of S-layers on solid supports or at the air-water interface have been studied by electron microscopy [3, 4, 5, 6], scanning tunneling microscopy [10] and atomic force microscopy [8, 9]. Lattice constants have been obtained but the resolution has not been high enough to determine the detailed shape and size of the proteins. X-ray crystallography has been utilized to solve the protein structure in few cases in which three-dimensional crystals of S-layer proteins have been obtained.
Small-angle X-ray scattering, SAXS, is a powerful method for determining the low resolution shape of proteins or to study aggregation of proteins in solution. Recently, Pavkov et al. [10] determined the high resolution structure of the N-terminal domain of SbsC of *Geobacillus steareothermophilus* in a three-dimensional crystal using X-ray crystallography. They used also SAXS to determine the rod-like shape of the C-terminal region of SbsC. The organization of S-layer proteins of Gram-positive *Clostrium difficile* was also studied using crystallography and SAXS [11]. The *C. difficile* S-layer consists of low- and high-molecular-weight S-layer proteins. A truncated mutant of the low molecular weight S-layer protein was crystallized and the three-dimensional structure was determined with the resolution of 2.4 Å. The shapes of the low-molecular-weight S-layer protein, both full length and the crystallized truncation mutant, and a non-covalent complex of low- and high-molecular-weight S-layer protein were determined using SAXS. Both the protein and the complex were elongated in shape.

The S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287, a Gram-positive bacterium with potentially probiotic properties, consists of 435 amino acids and has a molecular weight of 46 kDa. Precipitation in aqueous solution and cell wall binding of recombinant SlpA (rSlpA) truncation mutants was studied by Åvall-Jääskeläinen et al [12]. The truncation mutants comprising amino acids 190-423 formed a precipitate, while truncation mutants consisting of amino acids 1-145, 1-189 or 1-290 did not form a precipitate in the solution. The truncation mutants containing N-terminal amino acids 1–145 were found to attach to the cell wall while the truncation mutants without N-terminal amino acids did not bind to the cell wall. The N-terminal part of SlpA was shown to bind a polysaccharide receptor within the cell wall. Self-assembly products of rSlpA truncation mutants were studied using transmission electron microscopy (TEM). The truncation mutants of rSlpA consisting of amino acids 179–435 (rSlpA179–435) formed crystalline self-assembly products similar to that formed by full length SlpA while rSlpA190–435, rSlpA167–435 and rSlpA149–435 did not form regular lattice structures. This lead to the conclusion that amino acids 179–435 are responsible for *L. brevis* S-layer self-assembly [12].

The purpose of this work was to study the behaviour of the self-assembly domain containing truncation mutants of *L. brevis* surface layer proteins rSlpA179–435 and rSlpA167–435 in aqueous solution to obtain information on the protein-protein interactions that would be important for formation of the S-layer. These structural domains have appeared to be more soluble than the full length SlpA and the smaller of them, rSlpA179–435, formed a crystalline precipitate with about the same structure as the full length SlpA.[12] The same 4-5 mg/ml protein solution was studied using both SAXS and transmission electron microscopy (TEM), although the solution was diluted into a concentration of 1 mg/ml for TEM.

2. Experimental

2.1. Preparation of samples for SAXS

Sample 1. 1.3 mg of rSlpA (residues 167–435) in water was lyophilized o/n and dissolved in 10 mM Tris-HCl pH 7.5 to a final concentration of 4 mg/ml. The solution was centrifuged (16000 g, 20 minutes, +4 °C) and the resulting pellet was discarded.

Sample 2. 1.3 mg of rSlpA (residues 179–435) in water was lyophilized o/n and dissolved in 10 mM Tris-HCl pH 7.5 to a final concentration of 5 mg/ml. The solution was centrifuged (16000 g, 20 minutes, +4 °C) and the resulting pellet was discarded.

Sample 3. 1.3 mg of rSlpA (residues 179–435) in water was lyophilized o/n, dissolved in 5 M guanidium hydrochloride (denaturation) and dialyzed against 10 mM Tris-HCl pH 7.5 o/n at +4 °C (renaturation). After dialysis the volume was adjusted with 10 mM Tris-HCl pH 7.5 to a final concentration of 5 mg/ml. The solution was centrifuged (16000 g, 20 minutes, +4 °C) and the resulting pellet was discarded.
2.2. Electron microscopy

The sample 1 was placed on EM grid (Quantifoil 400 mesh holey carbon) and vitrified using Vitrobot (FEI company). The excess water was removed by blotting the grid twice for 1 seconds and the sample was by plunged into liquid ethane/propane mixture. The sample was then imaged in 18 K temperature using JEOL JEM-3200FSC Liquid Helium Cryo-TEM with 300 kV acceleration voltage and field emission gun. Images were taken with a GATAN Ultrascan 4000 CCD camera with a pixel size of 14 µm.

The samples 2 and 3 were placed on EM grids (Quantifoil 400 mesh holey carbon) and vitrified using Vitrobot (FEI company). The excess water was removed by blotting the grid once for 1.5 seconds and the sample was by plunged into liquid ethane/propane mixture and then imaged in liquid nitrogen temperature using FEI Tecnai 12 electron microscope with 120 kV acceleration voltage and LaB$_6$ filament. The images were taken using GATAN Ultrascan 1000 CCD camera with a pixel size of 14 µm.

2.3. SAXS experiments

The sample 1 was measured at the beamline X33 at EMBL/DESY, Hamburg. The wavelength was 1.5 Å. The range of the magnitude of the scattering vector $q$ was from 0.0088 to 0.6 Å$^{-1}$. Here $q$ was defined as $q = (4\pi\sin\theta)/\lambda$, where $2\theta$ is the scattering angle and $\lambda$ is the wavelength. The intensity was recorded with a photon counting Pilatus 1M pixel detector (67 x 420 mm$^2$). The measurement time was 8 times 30 s. A solution of bovine serum albumin (BSA) (molecular mass 66 kDa) was measured for calibration of intensity at zero angle. The preliminary data treatment (masking, integration, correction for detector response) was done using the software at the beamline.

The SAXS measurements for samples 2 and 3 were performed using a conventional X-ray tube with CuK$_{\alpha}$ radiation (1.54 Å) monochromatized by using a collimating Montel multilayer (Incoatec). The point focus beam geometry was used. The intensity curves were measured with an HI-STAR area detector (Bruker AXS) at a sample-to-detector distance of 50 cm. The scattering vector scale was calibrated on the basis of the known diffraction peaks of silver behenate [13]. The range of $q$ was 0.02–0.42 Å$^{-1}$. The samples and their buffer backgrounds were measured for 2 hours each.

3. Results

3.1. Electron microscopy results

Electron micrographs of sample 1 (Figure 1) show that the rSlpA$_{167-435}$ proteins form globular particles in the solution. Similar particles were also seen in electron micrographs of samples 2 and 3. The seemingly longest diameters of 264 particles for sample 1, 400 particles for sample 2, and 160 particles for sample 3 were measured by hand. The mean particle sizes were 200 Å for sample1, 267 Å for sample 2, and 228 Å for sample 3. Histograms of the longest particle diameters for samples 1-3 are given in Figure 2. Most of the particles were isolated in the solution, but few large agglomerates were also seen. However, the number of such agglomerates was small and their effect on SAXS intensity curves was assumed negligible. The edges of the particles were darker than the central part in the electron micrographs, indicating that the central part of the particles is generally more sparse than the edges. Some of the largest particles could be separate particles overlapping in the image and it was difficult to see particles smaller than 75-150 Å, depending on the image. Thus, it is possible that there are particles smaller than those seen in the distributions present in the samples.
Figure 1. Electron micrograph of rSlpA\textsubscript{167–435} particles in solution (sample 1).

Figure 2a. Distribution of the longest distances measured from the rSlpA\textsubscript{167–435} particles in sample 1.

Figure 2b. Distribution of the longest distances measured from the rSlpA\textsubscript{179–435} particles in sample 2.

Figure 2c. Distribution of the longest distances measured from the rSlpA\textsubscript{179–435} particles in sample 3.
3.2. SAXS results

The SAXS intensity of sample 1 (Figure 3) obeyed Guinier law up to 0.022 Å⁻¹ and Porod law in the interval 0.22 Å⁻¹ < q < 0.27 Å⁻¹. The distance distribution function was computed from the SAXS intensity using the program GNOM [14, 15]. For sample 1 an excellent solution was obtained using the maximum particle dimension of 300 Å. The obtained $R_g$ value was 88 ± 2 Å. The volume of the particles was determined to be 350 nm³. The molecular weight of the rSlpA monomer is 28.61 kDa, which corresponds to the volume of 35.3 nm³, assuming that the protein density is 0.81 Da/Å³. This indicates that the average rSlpA₁₆₇–₄₃₅ particle in solution contains 10 monomers.

The intensities of samples 2 and 3 were almost equal (Figure 3), differing slightly from the intensity of sample 1 at low $q$ values. Distance distributions were also computed and for sample 2 an excellent solution was obtained using the maximum particle dimension of 250 Å. The obtained $R_g$ value was 80 ± 2 Å. This result corresponds well to the mean particle diameter obtained by EM. However, since EM shows that there are also larger particles, the longest diameter was varied on the basis of the EM results. For instance, another distance distribution function with the longest distance of 345 Å was also found to fit well to the experimental SAXS data (good solution). The corresponding $R_g$ value was 117 ± 5 Å, which was at the upper limit of what fit into the Guinier region observed in the intensity.

For the denatured/renatured sample 3, an excellent solution was obtained with a maximum particle dimension of 220 Å, with $R_g$ value of 75 ± 1 Å.

Figure 3. SAXS intensities of samples 1 (red), 2 (blue), and 3 (green).

Figure 4. Distance distribution function calculated from the intensity of sample 1.

Attempts were made to model the SAXS intensity of samples 1-3 with spherical particles or spherical shells of varying sizes and to solve the volume distribution using the program GNOM [14, 15]. No good fitting results were obtained for any of the samples.

4. Discussion

In previous studies it has been observed that the full length SlpA and the self-assembly domain truncation mutant rSlpA₁₇₉–₄₃₅ form aggregates readily in aqueous solution. However, the precipitation of the full length SlpA in solution is much stronger than that of the truncation mutants (U. Hynönen, personal communication) and the cell wall binding domain remains soluble without precipitation. The N-terminal cell wall binding domain is hydrophilic and carries a net positive charge and the self-assembly domain carries a negative net charge [12]. That is why it is of interest to study these domains separately in solution. In this study the behaviour of the self assembly domain truncation mutants rSlpA₁₆₇–₄₃₅ and rSlpA₁₇₉–₄₃₅ were studied in aqueous solution.

The proteins were observed to form aggregates, the largest diameters of which varied between 73 Å and 400 Å, with a mean particle size of 200 Å for sample 1 on the basis of EM results. There was more variation in the particle size of sample 2 than in the denatured/renatured sample 3 and sample 1.
SAXS intensities of all the samples resembled each other closely and no essential differences in the aggregation of the samples 1-3 were observed by SAXS. We were not yet successful in preventing the aggregate formation and are thus not able to determine the detailed shapes of the monomers. The outer regions of both rSlpA$_{167-435}$ and rSlpA$_{179-435}$ aggregates were darker than the central part in the electron micrographs, indicating that the central part may be less dense than the edges. This kind of attachment may arise from the natural property of the SlpA subunits to attach to each other both in aqueous solution and on the cell wall surface.

It is of interest that the aggregates of SlpA self-assembly domains are able to stay soluble regardless of their function to form a large, crystalline layer in the bacterial cell wall. The full length SlpA forms weakly crystalline precipitates in solution [16]. The self-assembly into crystalline structures is much more effective in the presence of cell wall [12, 16]. It may thus be energetically more favorable for rSlpA particles to form oligomeric aggregates rather than crystalline arrays in solution environment.

Unlike _L. brevis_ SlpA, S-layer protein domain responsible for S-layer self-assembly, rSbsC$_{447-1099}$ of _Geobacillus stearothermophilus_ [7] and low molecular weight S-layer proteins and a complex of low and high molecular weight S-layer proteins _Clostridium difficile_ [8] appear to be monomeric in aqueous solution. Both _G. stearothermophilus_ and _C. difficile_ S-layers require the presence of calcium to form S-layer while in case of _L. brevis_, calcium is not required for the S-layer formation. Thus, the formation of _L. brevis_ rSlpA$_{179-435}$ oligomers could be due to hydrophobic interactions between the protein monomers in aqueous solution.

S-layers are capable of forming crystalline coatings on the bacterial cell surface and at water-air interface [1]. We are also studying using SAXS the structure of S-layer of _Lactobacillus brevis_ directly on the intact cells and as reassembled arrays on purified _L. brevis_ cell wall fragments. All these samples have showed periodic order with a spacing of about 80 Å. [16, 17]. However, differences in the degree of order in S-layers on the intact cells and reassembled on cell walls were observed [16, 17]. These results indicate that the key point in the understanding and controlling the self-assembly of S-layer proteins are the intermolecular interactions both between the S-layer protein monomers and between S-layer proteins and the cell wall.

5. Conclusions
In a previous study [12] about the same oblique lattice was observed in the precipitates of the length rSlpA and the recombinant protein SlpA$_{179-435}$, but the precipitate of the slightly larger N-terminally truncated protein, rSlpA$_{167-435}$, did not show any regular lattice structure. According to SAXS and EM results, both rSlpA$_{167-435}$ and rSlpA$_{179-435}$ formed stable oligomers in aqueous solution, presumably via hydrophobic interaction. The oligomers of both truncation mutants showed similar variation of size and shape. SAXS curves were nearly equal, which indicates no significant change in protein conformation between the truncation mutants.

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