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PspA can form large scaffolds in Escherichia coli
**PspA can form large scaffolds in *Escherichia coli***

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**ABSTRACT**

The phage shock protein A (PspA) of *Escherichia coli* stabilizes the cytoplasmic membrane under stress conditions. Here we demonstrate that PspA can form hollow spherical or prolate spheroidal particles of about 30-40 nm diameter with a scaffold-like arrangement of protein subunits at the surface. The 'PspA-scaffold' is the basic structure that is common to all particles. The PspA-scaffold may be of fundamental importance, as it could allow PspA to stabilize the integrity of membranes through numerous contact points over a large surface area.

**keywords:** phage shock proteins, membrane stabilization, membrane stress

**Abbreviations:** EFTEM: energy-filtered transmission electron microscopy
1 INTRODUCTION

Stable trans-membrane ion gradients are a prerequisite for many biological processes, such as respiratory energy conservation, flagellum rotation, or substrate transport. Membrane stress can affect the ion gradients and thereby cellular viability. In *Escherichia coli*, membrane stress conditions that perturb membrane integrity or energetization have been found to induce the production of PspA. Such stresses include: the proliferation of the filamentous phage F1, the presence of 10% ethanol, hyperosmotic shock, heat shock [1], alkaline conditions at stationary growth phase [2], shock with n-hexane or cyclooctane [3], the inhibition of membrane protein insertion by depletion of YidC [4], or membrane stress related to defects in Tat-dependent protein translocation [5]. It has been further demonstrated that Sec- and Tat-dependent protein translocation can be enhanced by higher PspA levels [5-7]. The mechanism by which PspA contributes to membrane stability is still unclear, but PspA - despite not having a trans-membrane domain on its own - is able to interact with membrane lipids [8] as well as with the membrane-integral proteins PspB and PspC, both encoded by the *pspABCDE* operon [9]. PspA is a bifunctional protein that also affects transcription of its own operon by interaction with the transcriptional activator PspF, which is encoded upstream of *pspA* by a divergently transcribed gene [10,11].

So far, PspA has been shown to be able to form symmetric rings of about 1 MDa in size [12]. Here we report the first detergent-free purification and ultrastructural characterization of *in vivo-*folded PspA from a membrane fraction. Native PspA can form a large and regular scaffold structure, which self-interacts to form spherical or prolate spheroidal extended superstructures. The scaffold is the conserved structure and the variable superstructures are likely to be caused by fragmentation of larger scaffolds during cell disruption. These results support the view that PspA may contribute to the maintenance of membrane integrity in stressed cells by multiple interactions to large surface areas.
2 MATERIALS AND METHODS

2.1 Strains and plasmids

*E. coli* strain XL1-Blue Mrf' Kan (Stratagene) was used for all cloning steps and MC4100 [13] was used for expressions, unless stated otherwise. For studies with $\text{P}_{\text{araBAD}}$-dependent expression, an arabinose-resistant MC4100 derivative was generated [14]. The $\Delta\text{pspA}::\text{kan}$ strain JW1297 and its parental strain BW25113 were used for complementation analyses [15]. *E. coli* was grown at 37°C on LB medium (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract) in the presence of the appropriate antibiotics (100 µg/ml ampicillin, 20 µg/ml chloramphenicol). In some experiments, the pH of the cultures was adjusted at indicated time points to pH 9.0 by addition of 50 mM Tris HCl pH 9.0 (at 37°C). The gene encoding PspA was amplified by PCR using the primers PspA-NdeI-F (5'- ACA ACC ATA TGG GTA TTT TTT CTC GCT TTG c-3') and PspA-HindIII-R (5'- TAT TAA AGC TTG TCT TGC TTC ATT TTG GCT T-3'), and cloned into the corresponding sites of pET22b+ (Novagen). The resulting pEX-pspA-H$_6$ vector encodes a C-terminally His$_6$-tagged PspA. The pspA-H$_6$ fragment was excised with Xbal / Scal and cloned into pTB-DG [16], resulting in pEX-pspA-H$_6$-tac for expression under control of P$_\text{tac}$. For expression under control of the $\text{P}_{\text{araBAD}}$ promoter, pspA-H$_6$ was amplified from pEX-pspA-H$_6$ by PCR using the primer PspA-NcoI-F (5'- ATA TAC CCA TGG GTA TTT TTT CTC GCT TTG C-3') and PspA-XbaI-R (5'- CTA GTT AGA TCT CAG CGG TGG CAG CAG CCA A C-3'), restricted with NcoI and XbaI and cloned into the corresponding sites of pBAD22 [17], resulting in the expression vector pBAD-pspA-H$_6$. In pBAD-pspA-strep, the C-terminal His$_6$-tag is exchanged at its XhoI site by a Strep-tag II from pTYB11 (IBA, Göttingen). All constructs were confirmed by DNA sequencing.

2.2 Biochemical methods

His$_6$-tagged PspA (PspA-H$_6$) was purified from cells that were resuspended in five volumes of 20 mM Tris HCl pH 8.0 containing 75 mM NaCl and 5 mM imidazole (buffer A) and passed twice
through a French Press cell at 138 MPa. After removal of cell debris (10 min, 15,000 x g, 4°C), membranes were sedimented (130,000 x g, 1h, 4°C) and resuspended in buffer A to 2 mg protein / ml. Membranes were then loaded on a Ni-NTA resin (Novagen), using buffer A for equilibration, loading and washing steps, and the same buffer with 250 mM imidazole (buffer B) for elution. Strep-tag II tagged PspA (PspA-Strep) was purified by affinity chromatography on Strep-Tactin Superflow™ columns (IBA) according to the supplier’s protocol, with the exception that 75 mM NaCl was used in washing and elution buffers. Detergent concentrations were as described for Ni-NTA affinity chromatography.

Proteins were separated by SDS-PAGE, semi-dry blotted and PspA was detected by anti-His<sub>6</sub>-Tag antisera obtained by rabbit immunization with either purified hexa-histidine-tagged HiPIP [18] or with purified hexahistidine-tagged PspA. For sucrose gradient centrifugation, a 100 µl sample was applied on a linear 4.4 ml 10-50% (w/v) sucrose gradient in 20 mM Tris HCl pH8.0, and centrifuged for 16 hours at 25,000 rpm in a swing out rotor (Beckman SW55 Ti). The gradient was thereafter fractionated in 350 µl fractions beginning from the surface. Proteins were precipitated by 15% (w/v) TCA and analyzed by SDS-PAGE and immunoblot-analyses.

2.3 Electron microscopy

Aliquots of affinity- and sucrose gradient-purified PspA were diluted to 70 µg/ml. PspA particles were adsorbed onto 15 nm carbon foil and negatively stained with 4% (w/v) uranyl acetate, as is described elsewhere [19]. Samples were analyzed at global bright-field settings using energy-filtered transmission electron microscopy (EFTEM) (CEM902, Zeiss; settings: energy-slit width = 25 eV, objective aperture = 30 µm, condenser aperture = 300 µm) at x 50,000 with 80 kV acceleration voltage [20]. Image registration was done with a cooled 1024 x 1024 CCD camera (Proscan CCD HSS 512/1024; Proscan Electronic Systems).
3 RESULTS

3.1 Functional PspA assembles into multimeric detergent-sensitive complexes

PspA is an unusual bifunctional protein that stabilizes membranes under manifold stress situations and in addition is involved in the regulation of its own operon [21]. It is a predicted coiled-coil-forming protein that can be detected in both membrane and soluble fractions [1,10]. PspA preparations obtained from detergent-solubilized aggregations have been shown to contain rings with a ninefold symmetry [12]. To analyze PspA from preparations that avoid detergent treatments, we first constructed an expression system that prevented the aggregation obtained with T7-promoter based expression systems (Figure 1A). Using a tightly regulated araBAD promoter-based system and 0.1% (w/v) arabinose for induction, less PspA was produced and the largest portion of PspA sedimented with the membranes, whereas only little PspA was found in inclusion bodies or in the supernatant.

The functionality of the recombinant PspA as produced by this vector system was assessed by complementation of a well-described ΔpspA phenotype, the viability during stationary growth under alkaline conditions [2]. A parental pspA wild type strain and its ΔpspA derivative, both containing the empty vector pBAD22, served as positive and negative controls, respectively. Arabinose was added to all cultures during late exponential growth, and the pH of the medium was adjusted to pH 9.0 at this point in time. As shown in Figure 1B, the ΔpspA strain was significantly more sensitive to this stress than the corresponding wild type strain. Expression from pBAD-pspA-H₆ fully complemented this phenotype, indicating that the recombinant PspA is physiologically functional. PspA induction in the tested wild type and pBAD-pspA-H₆-containing strains, as well as the absence of PspA in the pspA deletion strain were confirmed by Western blot using PspA-specific antibodies (Figure 1C).

Hexahistidine-tagged PspA could be purified from the membrane fraction without the use of detergents by affinity chromatography and a subsequent sucrose gradient centrifugation. Some
membrane vesicles that were identified by electron microscopy (data not shown) co-eluted with PspA during affinity chromatography and caused some impurity of the elution fractions (Figure 1D), but these could be separated in the sucrose gradient, in which membrane-free pure PspA formed a distribution maximum near 30% (w/v) sucrose (Figure 1E).

Purified PspA was sensitive towards the detergent CHAPS. Additions of CHAPS to a final concentration of either 0.2%, 0.5%, 1.1%, or 2.0% caused the disassembly of PspA in a concentration-dependent manner, resulting in a PspA fraction which banded near 10% (w/v) sucrose in sucrose gradient centrifugations (Figure 2). The concentration of 1.1% CHAPS corresponds to the concentration used for inclusion body solubilization during sample preparation for the reported structural single-particle analysis [12].

3.2 The PspA complexes are homomultimeric clathrin-like networks

Purified PspA sedimented under conditions usually used to prepare membranes and could be recovered from the pellet in a soluble form (data not shown), indicating that native PspA must form very large complexes. We therefore analyzed the structure of PspA by transmission electron microscopy. For initial experiments, purified PspA-His$_6$ was adsorbed onto Formvar film and detected by a monospecific anti-His-tag antibody and immuno-gold conjugates. When the protein was negatively stained with 2% phosphotungstic acid the procedure resulted in a close association of intensively cross-linked PspA particles that were densely labeled with gold conjugates (Figure 3).

In order to get ultrastructural data of individual particles, PspA particles negatively stained with uranyl acetate were analyzed by high resolution EFTEM in the elastic bright-field mode. PspA particles that banded at 30% (w/v) sucrose proved to be homogeneous. These particles appeared as spherical or short peanut-shaped extended structures (Figure 4). The spherical particles had an average diameter of $37.2 \pm 5.5$ nm ($n = 144$; minimum diameter 24.9 nm, maximum diameter: 56.2 nm), and occasionally showed gaps or holes in the structure due to the
lack of subunits or disrupted surfaces that suggested a hollow inner space (single arrows in Figure 4A,B). Independent of their general shape and size and without the use of averaging techniques, all individual particles showed a distinct ‘clathrin-like’ surface pattern - a large scaffold formed by PspA. This conserved PspA-scaffold thus can be regarded as the principle structure of PspA (Figure 4B: circles). In this scaffold, globular masses (Figure 4B: arrowheads) are often linearly aligned with a line-spacing of about 6 nm (Figure 4B: branched arrow). They are interconnected by thin structures that may be formed by the predicted coiled-coils of PspA. The observed PspA structures were independent from the His\textsubscript{6}-tag, since a Strep-Tag II tagged PspA led to the same particle morphology (data not shown).

4 DISCUSSION

PspA plays an important role in the stabilization of membrane integrity and is induced in its synthesis by several different membrane stress situations. However, the mode of PspA action remains to be resolved. Since PspA is predicted to consist mainly of coiled-coil structures, it is likely that PspA functions by forming an unusual structure that somehow can stabilize membranes. By avoiding detergent treatment during purification, we now show that PspA can form very large networks that can build up spherical or prolate spheroidal, peanut-like structures. The unifying characteristic of all these differently shaped structures is a scaffold. The various superordinated spherical or prolate spheroidal structures may well be a result of self-interactions of fragmented, much more extensive PspA scaffolds. In contrast, the scaffold structure of PspA is very likely to be physiologically relevant, as it constitutes all of the differently shaped particles seen in electron micrographs. In the scaffold, the predicted large coiled-coil domain in the central region of PspA might bridge the distances from one node to the next. Most likely, such a coiled-coil will allow the formation of antiparallel PspA dimers that could serve as the ‘minimal unit’. One might speculate that the ‘head domains’ of these units mediate the interactions that are required
for scaffold formation, but these aspects need further investigation. The network structure may under certain assay conditions be in an equilibrium with the ring structures and aggregates that have been observed in preparations that applied CHAPS for initial solubilization steps [12]. The reported ring structure has an outer diameter of 20 nm and thus is smaller than the scaffold particles that we report here and resemble partially disassembled scaffold-particles that we observed after CHAPS treatments (data not shown).

The structural data support the idea that PspA scaffolds may physically stabilize stressed membranes by multiple interactions over large surface areas. Such large scaffolds could support membrane integrity even better than a ring could do and promote the closure of transient 'holes' or leaks by preventing their extension. Multiple membrane interactions of the PspA scaffold could stabilize a flat conformation of the scaffold at the membrane, which then could cover large membrane regions. Alternatively, PspA might be able to form longer rod-like structures, such as observed under certain conditions with the PspA homologue in plastids, VIPP1 [22]. In analogy to VIPP1, the physiologically important assembly status of PspA might be controlled by the activity of ATP-hydrolyzing chaperones [22]. The structure of our purified PspA particles strongly resembles that previously observed with VIPP1 [23]. The resolution in the published VIPP1 electron micrographs did not reveal the ultrastructure of these particles, but their dimensions and the different shapes apparently coincide between PspA and VIPP1. Circular structures have been described, but VIPP1 could also form bipartite assemblies and even higher ordered extended structures [23]. As VIPP1 functions in the generation and possibly in the stabilization of thylakoid membranes, the mode of membrane interaction may be similar for VIPP1 and PspA [24,25]. The structural analogy to the vesicle scaffold protein clathrin and the reported functions of VIPP1 and PspA in thylakoid membrane formation and cytoplasmic membrane stabilization suggest that PspA scaffolds may generally serve to support membranes.
5 ACKNOWLEDGEMENTS

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6 REFERENCES


7 FIGURE LEGENDS

Figure 1: Production of functional PspA. (A) Analysis of expression of \textit{pspA} from pEX-\textit{pspA-H}$_6$ (T7 promoter) in \textit{E. coli} strain BL21 DE3 (left, SDS-PAGE) or from pBAD-\textit{pspA-H}$_6$ (\textit{araBAD} promoter) in strain MC4100 (middle: SDS-PAGE; right: immunoblot, His$_6$-Tag-detection); "T" total lysate, "IB" inclusion bodies, "S" soluble proteins "M" membrane fractions. (B) Complementation of the alkaline pH sensitivity during stationary growth of a \textit{ΔpspA::kan} strain by PspA as produced from pBAD-\textit{pspA-H}$_6$. The columns show the optical densities of the cultures (OD$_{600nm}$) at the beginning of the stationary growth phase (induction) and 21 hours after induction. Error bars are standard deviations as calculated from five independent experiments. wt: parental strain BW25113 / pBAD22, \textit{ΔpspA}: BW25113 \textit{ΔpspA::kan} / pBAD22; \textit{ΔpspA} + PspA: \textit{ΔpspA} / pBAD-\textit{pspA-H}$_6$. (C) Western blot analysis of PspA levels in the cultures used for the complementation analyses described in (B). PspA was detected with polyclonal antibodies raised against purified His$_6$-tagged PspA. Positions of marker proteins (in kDa) are indicated. (E) Purification of PspA by Ni-chelate affinity chromatography without detergent treatment. Fractions were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue-staining. S: soluble proteins, M: membrane fraction, F: flow-through (unbound material), W: wash-fraction, E1-7: elution fractions. (F) Sucrose gradient of protein purified in (E), showing that PspA forms a distribution maximum near 30% (w/v) sucrose. Some PspA sediments with the membrane fraction to the bottom of the gradient.

Figure 2. CHAPS treatment results in a dissociation of large PspA multimers. PspA as purified without the use of detergents or chaotropic salts was mixed with indicated concentrations of CHAPS and subsequently analyzed by sucrose gradient centrifugation. The gradient fractions were analyzed by SDS-PAGE and PspA was detected by Western blotting. The PspA band on the top of the gradient results from the CHAPS treatment.
**Figure 3.** Identification of PspA particles in PspA preparations by immuno-gold labeling. The His<sub>6</sub>-Tag of PspA preparations was detected by specific polyclonal antibodies raised against His<sub>6</sub>-tagged HiPIP (see methods), which in turn were detected by gold-labeled antibodies (10 nm gold particles).

**Figure 4.** Negatively stained PspA particles. (A) Survey view, 30% (w/v) sucrose fraction; branching arrows depict bipartite particles with pronounced contact sides; the arrow points to a 'hole' in the structure. (B) Close-up views of single, bipartite and tripartite particles. The arrow designates a 'hole' in the structure; arrowheads depict mass units (mu); branched arrows depict rows of mass units; circles designate clathrin-like arrangements of subunits.
Figure 1
Standar et al.

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Western Blot

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elution fractions

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10% sucrose gradient 50%

membrane-free PspA
membrane-attached PspA
Figure 2
Standar et al.

Sucrose gradient fraction

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10% sucrose

disassembled PspA
membrane-free PspA
membrane-attached PspA

PspA