Oligomerization, Membrane Anchoring, and Cellulose-binding Characteristics of AbpS, a Receptor-like Streptomyces Protein*

Stefan Walter‡ and Hildgund Schrempp
From the Fachbereich B Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany

Streptomyces reticuli produces a 34.6-kDa surface-anchored protein (AbpS) whose surface-exposed N terminus binds strongly to Avicel, a dominantly crystalline type of cellulose. The generation of a large set of mutated abps genes and the subsequent analysis of the corresponding proteins in vitro as well as in vivo in a Streptomyces host allow the assignment of the following characteristics for AbpS. (i) Amino acid residues participating directly in the cellulose-interaction are located at the N terminus. (ii) As ascertained by cross-linking experiments, AbpS forms homotetramers in its soluble as well as cellulose-bound form. (iii) The intermolecular assembly of four AbpS molecules is governed by two transmembrane domains (including amino acids 60–110 and 161–212). Both domains possess large portions of α-helical regions in which hydrophobic amino acids are located on one side as known from coiled-coil proteins. (iv) The C-terminal part of AbpS comprising 35 amino acids contains a transmembrane domain. Due to the surface-exposed N terminus of AbpS and the presence of transmembrane helix the C terminus has to be situated in the cytoplasm of the S. reticuli hyphae. Thus AbpS connects the interior of the mycelia with the extracellular space and binds cellulose using a unique cellulose-binding module.

Streptomyces are aerobic Gram-positive soil bacteria, which are adapted optimally to their natural environment (1). They undergo a complex process of morphological differentiation and growth, characterized by the formation of substrate mycelia, followed by a phase of aerial growth. Within the hyphae, chains of spores are formed (2–4). Their resistance to heat, dryness, or cold ensures survival of the strains in unfavorable periods (5). The production of anti-microbial substances may inhibit growth of competing organisms (6). The utilization of biopolymers (i.e. cellulose, chitin, or xylan) is a typical characteristic of streptomycetes, as they possess a wide variety of the corresponding catabolic enzymes (7). Additionally Streptomyces hyphae are surrounded by cell walls, which protect them from osmotic or mechanical disruption and extracellular attacks. Murein (peptidoglycan) is the main cell wall polymer, consisting of glycan strands (β 1–4-linked N-acetylglucosamine and N-acetylmuramic acid) and oligopeptides. These stem peptides are linked to the carboxyl group of N-acetylmuramic acid and interconnect glycans molecules via an interpeptide bridge. Peptidoglycan acts as permeability barrier, which should also be flexible, permitting morphological changes and allowing transport out of and into the mycelia (for reviews see Refs. 8 and 9). In addition, teichoic acids are covalently linked to muramic acid residues in peptidoglycan (cell wall teichoic acids) and may constitute 60% of the cell-wall material. The physiological role of the cell wall teichoic acids is thought to be involved in ion exchange, keeping the peptidoglycan sacculus in an expanded state by charge repulsion and control of the activity of autolytic enzymes. Other functions are related to phase binding and immunogenicity (10, 11). In addition to the teichoic acids, proteins have been found to be associated with the cell wall. In Streptomyces species only few surface-exposed proteins have been described up to now. These include a 23-kDa protein from Streptomyces lividans (12), a cell-bound esterase synthesized by the cyclophilin A-producing strain Streptomyces chrysomallus X2 (13), the mycelia-associated cellulase (14), catalase-peroxidase from Streptomyces reticuli (15), and the surface-active proteins (i.e. SapB from Streptomyces coelicolor and its homologue from Streptomyces tendae), which are involved in erecting aerial hyphae (16).

Recently we identified a 35-kDa protein from S. reticuli, which is very likely covalently anchored to the cell wall (17). Its N-terminal part protrudes from the surface of the hyphae, as demonstrated by immunolabelled ultrathin sections and investigations by electron microscopy (18). The protein has no enzymatic activity, but it interacts strongly with crystalline forms of cellulose (Avicel). AbpS1 (for Avicel-binding protein from S. reticuli) recognizes other biopolymers merely weakly (chitin and Valonia cellulose) or not at all (xylan, starch, and agar). By comparing the deduced AbpS sequence, no homology was found to any discovered cellulose-binding domain, which were often present within cellulases (19). AbpS possesses an up to now unique cellulose-binding module. By analysis of the secondary structure of the deduced AbpS sequence, a large centrally located α-helical structure showing a weak homology to the tropomyosin protein family and the streptococcal M-proteins (20) was identified. As AbpS has also been found to be associated to protoplasts, it is predicted that a C-terminally located stretch of 18 hydrophobic amino acids anchors the protein to the cytoplasmic membrane.

Beside streptomyces, surface proteins possessing diverse functions were also discovered in several other bacteria (21). In pathogenic bacteria streptococci and staphylococci (22–24) these proteins are often called microbial surface components recognizing adhesive matrix molecules (MSCRAMM), such as fibronectin, collagen, or immunoglobulins (25, 26). Because of their involvement in adherence of the bacteria and concea-

* This work was supported in part by the Sonderforschungsbereich 431. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Universität Osnabrück, FB Biologie/Chemie, Barbarastrasse 11, 49069 Osnabrück, Germany. Tel.: 49-541-969-2287; Fax: 49-541-969-2804; E-mail: Stefan.Walter@uni-osnabrueck.de.

1 The abbreviations used are: AbpS, Avicel-binding protein from S. reticuli; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; N-I-NTA, nickel-nitrilotriacetic acid; PBS, phosphate-buffered saline; DSP, dithiobis(succinimidyl propionate).
ment of the bacterial surface from the host's defense system, MSCRAMMs were studied intensively. Surface-associated heparin-binding proteins are frequent among pathogenic mycobacteria (27, 28). Various types of cell-wall-anchored proteinases are encountered in different genera of Gram-positive bacteria (29–31).

In this study we elucidate the detailed characteristics of the up to date unique Avicel-binding protein from S. reticuli. The investigations as to the membrane anchoring and complex formation support the conclusions that AbpS connects the Streptomyces cytoplasm with the extracellular environment and functions as a cellulose-receptor.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Transformations, and Cultivation—The wild-type strain S. reticuli Tu45 described by Wachhuser et al. (32) was obtained from H. Zahné, Tübingen, Germany. It was cultivated in pH-stable medium (MM3) supplemented with a carbon source (1% w/v), as outlined previously (33). For protoplasting, S. lividans 66 mycelia (provided by D.A. Hopwood, John Innes Institute, Norwich, UK) was transformed with plasmid DNA using the CaCl2 method (34) or its derivatives containing truncated abpS genes by standard protocols (35). PCR-based transformation of a 3.2-kb genomic SalI DNA fragment from S. reticuli, on which the complete abpS gene is located, was described earlier (17, 18). The DNA sequence of abpS is available in the EMBL data bank under the accession number X97071. The E. coli vector pET21a (Novagen, Madison, WI) was used as cloning vector for truncated forms of the abpS gene. The derivatives of pET21a containing the complete abpS gene or truncated genes with 5′ end deletions (resulting in N-terminally shortened AbpS-proteins with molecular weights of 32.3 kDa, 29.1 kDa, and 23.5 kDa) were described earlier (17). E. coli DH5α or the chloramphenicol resistant E. coli BL21 (pLyS8) (Novagen) was transformed with plasmid DNA using the CaCl2 method (35).

Polymerase Chain Reactions and Construction of Plasmids—The PCR reaction mixture contained different primer combinations: 10 ng of pUS1 (36). For self-ligation of the PCR products, in-frame SauI sites were incorporated into the primer sequences. For amplification of DNA with the primers S1 (GAGCATCTCGTCGACGTTCGTCAG) and S3 (TGCGCGGTGTCGACCTGGGC) and as template pUS1 (36) was used to substitute the SauI digested with SauI and NdeI-digested PCR products were ligated in-frame into the vector pET21a (Novagen) as outlined previously (33). After protoplasting, S. reticuli (containing the P3 (TGCGCGGTGTCGACCTGGGC) and as template pUS1 digestion was performed with the help of the T7 sequencing kit and Cy5-labeled standard primers (Amersham Biosciences).

4 Purification of Truncated His-tagged AbpS Proteins from E. coli or S. lividans—The E. coli BL21 (pLyS8) transformants containing the designed constructs (see above) were grown at 37°C in SOC medium (20 g of Bacto-Trypton, 5 g of yeast extract, 0.5 g of NaCl, 0.18 g of KCl, and 20 ml of 1 × glucose/liter, which had been supplemented with chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml)). Isopropyl-β-D-thiogalactopyranoside (final concentration 1 µM) was added when A600 had reached 0.6. After further 3 h of cultivation, the E. coli cells were harvested, washed, resuspended in sonication buffer (0.1 M NaHPO4, 0.01 M Tris-HCl (pH 8.0), and 8 µM urea), and disrupted using a Branson sonifier B12 for 3 min in 20 s intervals. Having removed the cell debris, N2O–NTA (Qiagen) was added to bind the His6 fusion protein. Unspeciﬁc bound proteins were removed by consecutive washings with buffer (0.1 M NaHPO4, 0.01 M Tris-HCl (pH 6.3), and 8 µM urea) containing 25 mM imidazol. The immobilized proteins were renatured by washing with buffers containing decreasing concentrations of urea (8-0 M) in a period of 2-3 h. The fusion protein was subsequently released by the addition of 0.5 M imidazol.

S. lividans transformants were grown in pH-stable medium (MM3) supplemented with 1% glucose for 48 h. After harvesting the mycelia by centrifugation, they were disrupted and the His6-tagged proteins were isolated as described above with or without urea-containing buffers. Isolation of Murine-associated AbpS from S. reticuli—Anti-AbpS antibodies had been gained previously (18). The IgGs were purified from a murine HICorporate serum (17) according to the supplier’s instructions. Subsequently, the purified IgGs were coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences) as described in the corresponding manual.

The murein layer was isolated from S. reticuli hyphae grown in 1 liter of minimal media as described earlier (18). Murein was treated with buffer (25 mM Tris-HCl (pH 8) and 20 mM EDTA) comprising 5 mg of lysozyme (Roche Applied Science) per ml and incubated at 37°C for 3 h. After centrifugation at 14,000 × g for 30 min, the AbpS-containing supernatant was mixed with immobilized (see above) anti-AbpS antibodies. After incubation for 3–5 h under gentle stirring, the Sepharose-AbpS complex was washed 3 times with 50 ml PBS (80 g of NaCl, 2 g of KCl, 2 g of KH2PO4, 11.5 g of Na2HPO4, and 0.01 M Tris-HCl (pH 7.4)) followed by 3 washes with 50 ml PBS. Finally the proteins were transferred onto nylon membranes, which were incubated in PBS containing the primary antiserum (1:100,000 dilution), the anti-His6 antibodies, or the anti-RGSHis6 antibodies (both 1:10,000 dilution) (Qiagen). After three washes, the blot was incubated in Cy5-labeled goat anti-rabbit IgG or mouse anti-rabbit IgG (Dianova, Hamburg, Germany). Color development was performed as described by West et al. (38).

Separation of Cell Compartments—S. lividans mycelia were harvested by centrifugation, washed 3 times with 10.3% sucrose, and murein was hydrolyzed by incubation with 3 mg of lysozyme ml-1 4 mg-1 wet weight of mycelia (35). After filtration the proteolysates were resuspended in water supplemented with 5 mM EDTA to destroy them osmotically. The membranes and associated proteins were separated from soluble proteins by ultracentrifugation (250,000 × g for 45 min). Membrane and cytoplasmic fractions contained about 50% of total cell proteins. The gel Electrophoresis and Western Blotting—SDS-PAGE was performed with 10% polyacrylamide gels in the presence of 0.1% SDS (37). If desired, proteins were transferred onto nylon membranes, which were incubated in PBS containing the primary antiserum (1:100,000 dilution), the anti-His6 antibodies, or the anti-RGSHis6 antibodies (both 1:10,000 dilution) (Qiagen). After three washes, the blot was incubated in Cy5-labeled goat anti-rabbit IgG or mouse anti-rabbit IgG (Dianova, Hamburg, Germany). Color development was performed as described by West et al. (38).

Urea-gradient PAGE—The lower polyacrylamide-gel (in a concentration of 4% and without SDS) was poured sidelong into the glass plate in gel casting stages, containing 12.5% polyacrylamide. After polymerization, the standard upper gel was overlaid so that one lane was formed. The proteins were loaded with a native loading buffer (36), and the electrophoresis was done at 15 mA to avoid heating above 25°C. Finally the proteins were stained with Coomassie Brilliant Blue.
Avicel-binding Protein AbpS

FIG. 1. Comparison of truncated AbpS proteins. A, the amino acid sequence (given in one letter code) of the wild-type protein is compared with the amino acid sequence of the truncated forms. The corresponding molecular masses are indicated as part of their denomination. B, the predicted secondary structure of AbpS and its truncated forms is shown in the same order above. α-helical structures are marked by shaded boxes, β-sheets by black boxes. The predicted hydrophobic segment is marked as TM. C, purification of AbpS. AbpS isolated from S. reticuli wild type was applied onto a SDS-PAA gel and after electrophoresis stained with Coomassie Blue. D, purification of the full-length and truncated AbpS-His6-tagged fusion proteins. All proteins were isolated from E. coli transformants carrying the respective plasmid constructs and tested for purity and quantity by SDS-PAGE and Coomassie Blue staining. The molecular masses of the standard proteins and the truncated AbpS forms (as part of their denomination) are given.

Cellulose-binding Assay—Proteins were incubated with Avicel (15 mg/ml) in 50 mM potassium phosphate buffer (pH 7) for 30 min. Avicel recovered centrifugation was washed 3 times with 50 mM potassium phosphate buffer containing 1 M NaCl. Avicel-bound proteins were released by heating in SDS sample buffer for 5 min at 100°C and subject to SDS-PAGE. Staining of proteins was done with Coomassie Brilliant Blue.

Detection of Protein-Protein Interactions—Total proteins or purified AbpS isolated from S. reticuli were denatured by SDS, separated on an SDS-PAGE, and subsequently transferred onto a polyvinylidene difluoride membrane (because of the cellulose-binding capability of AbpS, nitrocellulose membrane was avoided). The membrane was treated for 1 h with PBS (see above) containing 1% bovine serum albumin. The His6-tagged fusion proteins (which were to be tested for the interaction of the immobilized AbpS) were incubated with 1 M urea in small volumes for 1 h. Rapid renaturation was performed by diluting this solution 1:100 in PBS, which contained a piece of the nylon membrane with immobilized AbpS from S. reticuli. After incubation at 25°C the membrane was washed 3 times with 50 ml of PBS, and the fusion proteins that were trapped by the immobilized AbpS were detected immunologically by applying antibodies specific to a stretch of 5 histidines (for further details see “SDS Gel Electrophoresis and Western Blotting”).

Cross-linking of AbpS Complexes—Because of the absence of cysteine residues in AbpS the reactive bifunctional cross-linker dihydro(succinimidyl propionate) (DSP), which is cleavable by reducing agents, was chosen to interconnect subunits of protein complexes. For this purpose the concentration of each of the His6 fusion proteins (35.7, 27.1ΔC, 23.5ΔN, or 31.4ΔI) was adjusted to 10 μM. 3 μM (corresponds to 30 nmol) were diluted in 50 μl of buffer (25 mM Tris-HCl (pH 7)). The addition of 50 mM NaCl was found to inhibit ionic protein interaction, which increases the crosslinking specificity. To avoid intermolecular cross-linkages the molar ratio between DSP and denatured proteins (0.5% SDS) was adjusted so that no multimers were found. The molar ratio (DSP:protein) of 2:1 was found to be optimal. Therefore the cross-linking reaction was started by adding 2 μl of 10 mM solution of DSP (dissolved in MeOH). After 10 min at 30°C, the proteins were denatured by SDS at 95°C and analyzed by PAGE.

0.6 μg of AbpS isolated from S. reticuli were incubated with 10 mg Avicel in a final volume of 100 μl for 1 h. After 3 washes, 50 μl of 25 mM Tris-HCl (pH 7) was added to the Avicel, and subsequently the bound proteins were cross-linked as described above. As control, the same amount of unbound AbpS was used.

Circular Dichroic Spectroscopic Analysis—To assess the structure of AbpS or its His6 fusion variants (35.7, 27.1ΔC, 23.5ΔN, and 31.4ΔI) the concentrations of each of the proteins were adjusted to 0.2 mg/ml. The spectra (190–260 nm) were recorded at 25°C using a Jasco 610 spectrophotometer in a 0.1-mm path length cell. Data were recorded 5 times, and an average value was determined. The observed ellipticity [θ] (degrees) is converted in mean residues ellipticity by: [θ] = [θ] - 3298 Δe. Subsequently the data were analyzed by CD Spectroscopy Deconvolution program CDDN 2.1 (bioinfomatik, biochentech.uni-halle.de).

Reconstitution of AbpS—Soybean Azolectins (L-α-phosphatidylcholine solution obtained from Sigma) were washed twice with acetone in the presence of butylated hydroxytoluene and once with ether/vitamine E, dissolved in chloroform, and dried under a nitrogen stream. The liposomes were done according to Jung et al. (51). After solubilization of the liposomes by addition of 3% octyl β-D-glucopyranoside, the His6-tagged fusion proteins isolated from S. lividans were added and sonicated for 1 s and repeated 9 times. After removing the detergent by Bio-Beads SM-2 (Bio-Rad) the proteoliposomes were washed and concentrated by centrifugation at 250,000 × g.

RESULTS AND DISCUSSION

Creation of Truncated AbpS Variants—A PCR-based approach was followed to produce abpS genes with deletion at the 3′ end (31.0ΔC and 27.1ΔΔC) to complement a series of modified abpS genes that have shortened 5′ ends (18) (Fig. 1). After cloning of the genes the encoded proteins were predicted to be tagged with a valine, a glutamine, and six histidines at the C terminus. Internal deletions within the abpS gene were generated by inverse PCR with the pET21a derivative containing the complete abpS gene (resulted in the deduced proteins 31.4ΔI or 25.6ΔI). Each of the plasmids was introduced into E. coli BL21 (pLysS). Sequence analysis of the plasmids revealed that the designed reading frames were preserved at the religated sites and that no base exchange had occurred.

Each E. coli BL21 (pLysS) transformant harboring one of the different constructs was grown to the logarithmic phase, induced with varying concentrations of isopropyl-β-D-thiogalactopyranoside at different temperatures. Independently of the used conditions, each type of the fusion protein was found dominantly within insoluble inclusion bodies (more than 90%).
Therefore the proteins were bound to Ni$^{2+}$-NTA in the presence of 8 M urea. Removing urea stepwise (see “Materials and Methods”), the proteins were subsequently released by imidazol, and each type was found to have the predicted molecular weight (see Fig. 1D).

Identification of α-Helical Structures—To analyze whether the renatured truncated AbpS forms (isolated from E. coli) kept structural characteristics of the S. reticuli wild-type AbpS a circular dichroism spectrum of each of the proteins should be recorded. As requirement a purification method of AbpS from S. reticuli has to be established first. As reported previously (18) AbpS is covalently linked to the peptidoglycan layer of S. reticuli. Therefore, its murein was isolated as previously described (18). Subsequently, AbpS was released by the action of lysozyme and immobilized by Sepharose-coupled anti-AbpS antibodies. After elution, AbpS was obtained in a good degree of purity (Fig. 1C); however, only ~5 μg AbpS can be gained from 10 g mycelia (wet weight), grown in 1 liter culture.

Additionally the 35.7 His-tagged full-length AbpS fusion protein, the 23.5ΔN, 25.ΔI, and 31.ΔC forms were purified from E. coli as described (see “Material and Methods”). The concentration of each protein was adjusted to 0.2 mg/ml, and its CD spectrum was determined (Fig. 2A). Between 205 and 260 nm the spectra of all proteins were close to identical. Below 205 nm AbpS, isolated from its natural host (S. reticuli), showed a slightly higher difference in the absorption (Δε) value. Thereby, the deletion of different regions of the proteins did not significantly alter the overall structure of the protein. The competency for assembly of the secondary structure seems to reside in the composition of the protein sequence itself. The structural integrity is also reflected by the evaluated occurrence of 5 different protein-folding motifs (helix, anti- and parallel β-sheets, β-turns, or random coils). Their quantification as deduced from the CD spectra revealed that wild-type AbpS and its truncated forms consist predominantly of α-helical structures (ranging between 93 and 98.2%), and other structural motives are under-represented. This finding is in good agreement with a computer-supported prediction of the secondary structural elements within AbpS (Fig. 1B, scheme), comprising a large, centrally located α-helix flanked by two shorter helices, whereas the C-terminal helix is built by hydrophobic amino acids.

Fig. 2. Circular dichroic spectroscopic analysis. A, spectra from AbpS, isolated from S. reticuli, the His-tagged AbpS (35.7 kDa) (●), 23.5ΔN (∇), 25.ΔI (-×), and 31.ΔC (○) AbpS derivatives were acquired from 190–260 nm and given in relation to the difference in their molar absorbance (Δε). B, quantification of the occurrence of structural elements.

Fig. 3. Comparative in vitro and in vivo analysis of ΔCAbpSHis and AbpSHis. A, total proteins from S. lividans containing pWHM3 (lane 1, as control), pWA1 (lane 2), and pWΔC (lane 3) were separated with PAGE, transferred onto a nylon membrane, and immunologically analyzed by polyclonal anti-AbpS antibodies (left panel) or monoclonal anti-RGSH6 antibodies. The endogenous S. lividans AbpS is present in each of transformants and can be detected as ΔCAbpSHis and AbpSHis with anti-AbpS antibodies, but not with anti-RGSH6 antibodies, which each of the fusion proteins. B, the isolated proteins AbpSHis (left) and ΔCAbpSHis (right) were incubated with liposomes. The integration of the proteins was tested by purification of the proteoliposomes by centrifugation. The proteins (lanes 1), the unincorporated portion (supernatant of the first centrifugation step) (lanes 2), and the proteins in the proteoliposomes (lanes 3) were quantified by PAGE and Coomassie staining. C, S. lividans mycelia harboring the plasmid pWΔC were sonicated (as control in lane 1) or treated with lysozyme. After 1 h the proteins released by the digestion of the murein were separated from the protoplasts by centrifugation (lane 2). The protoplasts were burst by adding EDTA, and the soluble proteins (lane 3) were separated from the membranes (lane 4) by ultracentrifugation. The amount of the endogenous S. lividans AbpS and the C-terminal deleted form (ΔCAbpSHis) in each sample was determined after PAGE with the help of anti-AbpS antibodies. The individual volume applied onto the SDS-PAA gels was calculated in relation to the sample volume. D, mycelia of S. lividans containing pWHM3 (lane 1), pWA1 (lane 2), or pWΔC (lane 3) were sonicated in the presence (right panel) or absence (left panel) of urea. After purification of His-tagged proteins with Ni-NTA, the samples were analyzed by Western blotting (anti-AbpS antibodies) for the presence of co-purified S. lividans AbpS.
Figure 4. Identification of the interacting domains. A, protein-protein interactions. Immobilized wild-type AbpS was incubated with the indicated truncated His6-labeled proteins. Formation of the protein complexes was tested immunologically by using anti-His6 antibodies (lanes 1–9) and quantified by densitometrical evaluation. The values are given as percentages in reference to the protein amounts shown in lane 1. Immobilized AbpS incubated with none of the soluble proteins (lanes 9 and 10) were treated with the indicated antibodies and served as controls. B, AbpS and bovine serum albumin as control were dotted onto a polyvinylidene difluoride membrane and incubated with (lanes 1 and 2 and 1′ and 2′) or without (lanes 3 and 3′) the His6-tagged full-length AbpS form. After elution of the proteins from the membrane they were separated by PAGE. Instead, the use of anti-RGS6 antibodies allowed the specific detection of the modified proteins (Fig. 3A). AbpSHis or AbpSHis could be isolated in high purity with the help of Ni-NTA under denaturing conditions (Fig. 3D, right panel). After renaturation they were reconstituted into liposomes. Only AbpSHis was found to be integrated in the vesicles. Deletion of the hydrophobic part within ΔCAbpSHis led to inhibition of its integration into proteoliposomes (Fig. 3D). To study the membrane integration within the original host in more detail, S. reticuli membranes were isolated and the release of AbpS was tested. Only in the presence of SDS (1%) or Triton X-100 (1%) but not with EDTA, urea (up to 1 M), nor water, AbpS was unhinged from the membranes (data not shown). Therefore a membrane-association of AbpS by ionic forces or a budding of AbpS on the membrane-surface by hydrophobic interactions could be excluded.

Analyzing the Function of the C-terminal Hydrophobic Stretch of AbpS—In vivo AbpS was found to be associated with the membrane of the protoplasts, generated by removing the murein layer from the hyphae of S. reticuli (18). The C-terminally located hydrophobic helix was thus suspected to anchor AbpS to the membrane. To analyze this in more detail comparative in vivo and in vitro studies of AbpS with and without the hydrophobic segment were designed.

As a first requirement AbpS and the designed C-terminally truncated variant have to be synthesized in a Streptomyces host to guarantee native conditions, for example allowing the proper protein folding or membrane anchoring. Because there were no stable plasmids available for S. reticuli, the genetically best studied S. lividans strain was chosen as host. A disadvantage of S. lividans is the presence of an abpS homologue located within its chromosome (17). AbpS-negative S. reticuli or S. lividans mutants or AbpS-negative Streptomyces wild-type strains were not available.

Vector constructs (based on pWHM3) were designed having either the complete abpS gene (pWA1) or the 5′ end deleted abpS gene (pWAΔC) (encoding AbpS without the hydrophobic helix). They were kept under the transcriptional control of the upstream region of the S. reticuli abpS gene. In total, proteins of the transformants S. lividans (pWA1) or S. lividans (pWAΔC) each prolonged with a His6 tag (named AbpSHis or ΔCAbpSHis, respectively) could be found in addition to the endogenous AbpS homologue (see S. lividans (pWHM3) as control) with the help of anti-AbpS antibodies. Due to the similar molecular weight of the endogenous S. lividans, AbpS, and the plasmid-encoded His-tagged fusion protein, they could not be separated by PAGE. Instead, the use of anti-RGS6 antibodies allowed the specific detection of the modified proteins (Fig. 3A). AbpSHis or AbpSHis could be isolated in high purity with the help of Ni-NTA under denaturing conditions (Fig. 3D, right panel). After renaturation they were reconstituted into liposomes. Only AbpSHis was found to be integrated in the vesicles. Deletion of the hydrophobic part within ΔCAbpSHis led to inhibition of its integration into proteoliposomes (Fig. 3D). To study the membrane integration within the original host in more detail, S. reticuli membranes were isolated and the release of AbpS was tested. Only in the presence of SDS (1%) or Triton X-100 (1%) but not with EDTA, urea (up to 1 M), nor water, AbpS was unhinged from the membranes (data not shown). Therefore a membrane-association of AbpS by ionic forces or a budding of AbpS on the membrane-surface by hydrophobic interactions could be excluded.

Summarizing the data, the C-terminal helix was clearly identified as a transmembrane spanning segment. With simultaneous consideration that the N terminus of AbpS was previously found to protrude from the cell surface (18) and a transmembrane spanning helix is present, the C-terminal end of AbpS has to extend into the cytoplasm of the S. reticuli hyphae. Thus AbpS connects the interior of the mycelia with the extracellular space, where it binds insoluble cellulose. Such organization is reminiscent of the assembly of diverse classes of receptor proteins (39), often involved in signal transduction cascades.

To study the effect of the deletion of the transmembrane segment in vivo (in S. lividans mycelia), the overproduction of AbpSHis or ΔCAbpSHis in S. lividans was prevented by the cloning strategy (using the wild-type regulatory elements). As a result, AbpS and its derivatives were found in approximately the same concentrations (Fig. 3A) within the total proteins isolated from S. lividans containing pWHM3, pWA1, or pWAΔC, respectively.

It was expected that at least the C-terminally deleted protein ΔCAbpSHis would be released by digestion of the murein. But surprisingly the distribution of the endogenous S. lividans AbpS and ΔCAbpSHis in the different cell compartments (extra cellular, murein-associated, membrane-integrated, or cytoplasmic) was found to be equal (Fig. 3C). A possible cause for this in vivo effect, which contrasted the in vitro data, appeared to be the fact that the endogenous membrane-integrated S. lividans-
AbpS molecules form complexes with ΔCAbpSHis (plasmid-encoded) and impede, therefore, the release of the C-terminally deleted protein. The expected complex formation could be satisfactorily shown by co-purification of the endogenous *S. lividans*-AbpS (unable to bind to Ni-NTA) together with the His6-tagged AbpS derivatives (AbpSHis and ΔCAbpSHis) by Ni-NTA-based affinity chromatography (Fig. 3).

**Identification of AbpS Domains Required for Intermolecular Interaction**—To study the deduced interaction among AbpS molecules in more detail, total proteins (including the *S. reticuli* wild-type AbpS) from *S. reticuli* were denatured, separated by SDS-PAGE, and then immobilized on a nylon membrane. Subsequently identical amounts of the truncated and denatured AbpS forms were added to the membrane-immobilized AbpS in low salt buffer. The degree of intermolecular interaction was determined by immunological quantification of the membrane-retaining His6 fusion proteins (Fig. 4A). Independently of the presence (35.7 kDa form) or absence (31.4ΔN and 27.1ΔC) of the C-terminal part, each of the proteins strongly interacted with the immobilized *S. reticuli*-AbpS. However, continued deletions of the N-terminal part (32.3ΔN, 29.1ΔN, and 25.6ΔN) led to an increased loss of the binding ability. Therefore it can be concluded that the portion of the protein (including amino acids 60–110) situated at the beginning of the central α-helix plays an essential role in the complex forming process. The remaining binding level of the 23.5ΔN protein was attributed to a second domain identified by analyzing the characteristics of additional truncated forms. They extend from the middle (31.4ΔI) to the right side (25.6ΔI) of the centrally located α-helix (including amino acids 161–212). The 25.6ΔI form was found to retain only 15% of the maximal binding ability, which corresponded closely to that of the 31.4ΔI form (Fig. 4A).

As outlined above on the nylon-membranes (on which the separated cellular proteins from *S. reticuli* were immobilized), each of the added truncated AbpS forms were found to interact only with AbpS, demonstrating a high specificity of the protein-protein interaction. To verify this in more detail, the formed complexes were precisely cut out of the membrane, which was overlaid with the full-length His6-tagged fusion protein. Immunological identification clearly demonstrated the sole participation of AbpS and its His6-tagged form (35.7 kDa) in the intermolecular interaction (Fig. 4B). Based on these data, two domains responsible for AbpS-AbpS interaction were identified, matching regions predicted to contain only α-helical structures (Fig. 4A).

**Conditions for Intermolecular Interaction**—Mixtures of membrane-immobilized *S. reticuli*-AbpS and its His6-tagged full-length 35.7 kDa form were incubated under different conditions (20–90 °C or at pH 2.7) or treated with different amounts of chaotropic urea, leading to protein-denaturation protein (0.25–8 m urea) or increasing the ionic strength (1 M NaCl) (Fig. 4C). The protein complex was found to be stable in the presence of up to 1 M urea or 1 M NaCl. Because these conditions are unfavorable for ionic bonding, oligomerization based on the ionic strength could be excluded. At very low pH (2.7), high concentrations of chaotropic salt (commencing at 1 M) or high temperatures (higher than 50 °C) the protein-protein interaction was usually inhibited strongly. The above-described conditions for resolving the ability for complex formation exactly match those that inhibited the binding of AbpS to cellulose (17). These findings suggest that the *S. reticuli* AbpS interacts with cellulose only in an oligomerized form.

**Characterization of Oligomerization**—Using standard methods (sucrose gradients or gel filtration with various globular reference proteins) it was found that the molecular weight determined for AbpS neither correlated to that one for the AbpS monomer nor to any expected oligomer. This was attributed to the fact that the secondary structure of AbpS was found to be dominantly α-helical, governing an elongated shape of the protein (Figs. 1B and 2). Because correspondingly shaped reference proteins are missing, the mobilities of the various truncated AbpS forms and the *S. reticuli*-AbpS were comparatively studied in native PAA gels in the presence of increasing concentrations of chaotropic urea (0, 1.25, 3, and 5 M) (Fig. 5). The *S. reticuli* AbpS, the 35.7-kDa full-length AbpS-His6 fusion protein, and the 31.4ΔI protein (with a short internal deletion) were found to migrate into the gel only in the presence of 6 M urea. When applying to 3 M urea, no protein or only traces could be recovered. This effect was also ascertainable at low (5%) polyacrylamide concentrations. The 31.0ΔC truncated form possesses a deletion of 46 C-terminal amino acids, including the above-characterized hydrophobic membrane-integrated helix. The removal of consecutive 35 amino acids resulted in the 27.1ΔC truncated form. Both protein types were found to migrate into the gel containing up to 3 M urea. In comparison, the relative mobility increased under denaturing conditions (6 M urea). These data suggest that the homomultimeric protein complexes were formed by native conditions, whereas the presence of the hydrophobic segments provoked protein aggregation (Fig. 5).

Interestingly, the 23.5ΔN and 25.6ΔI proteins (both lacking one domain responsible for the intermolecular protein interaction) were monomeric under all conditions (0–6 M urea). This finding is in agreement with their low protein-protein interaction ability (Fig. 6). In contrast, the 32.3ΔN as well as the
29.1ΔN form, both possessing an increasing portion of a deleted N terminus, migrated already in the presence of 1.25 M urea as denatured protein complexes; this decreasing stability of the protein complexes correlates with their intermediate ability to interact with the wild-type AbpS (see Fig. 4A).

Again this finding indicates a protein-protein interaction mechanism, which governs the formation of a stable complex. Together with the structural information of AbpS a formation of coiled-coil multimers appears likely (Fig. 7). In coiled-coil proteins the α-helices provides hydrophobic amino acids to one side of the helix. This hydrophobic side is able to interact with the corresponding side of the next subunit. Ionic amino acids, proximate to the hydrophobic ones, direct the formation of dimeric, tetrameric, or higher organized complexes. Typical coiled-coil proteins are the dimeric tropomyosins (40), trimeric or tetrameric leucinezippers (41), or the pentameric Comp (cartilage oligomeric matrix protein) whose oligomerization domains marked similarities with proposed models of the pentameric transmembrane ion channels in phospholamban and the acetylcholine receptor (42).

Interestingly some M-proteins of the group A streptococci, which belong to the MSCRAMMs (see Introduction), have been shown to be coiled-coil dimers, appearing as fibrils on the bacterial surface in the electron microscope (23, 43, 44). The arrangement of the hydrophobic amino acids is also reflected in the primary sequence of a protein. Numbering the amino acids in a coiled-coil forming α-helix by “abcdefg” amino acids “a” and “d” were found to be hydrophobic (41). Analyzing the deduced AbpS protein sequence according to this rule, large protein sections (including amino acids 20 to 246) were identified with high potential for coiled-coil interactions (Fig. 7).

Constitution of the AbpS Complexes—To determine the constitution of the AbpS complexes, cross-linking experiments with the unspecific linking agent DSP were performed. For this...
The arrangement of hydrophobic amino acids. A, part of the deduced amino acid sequence of AbpS (from amino acid 20 to 246) is given in the one letter code. The arrangement of hydrophobic residues (AVLIPMFW, marked with gray boxes) according to the abedefg rule (given in the second line) of coiled-coil proteins is indicated by continuous boxes.

**Cellulose Recognition**—Comparative analysis of AbpS and each of the truncated forms revealed that a reduced oligomerization (29.1 ΔN, 23.5 ΔN, and 25.6Δ1) is accompanied with a decreased capability for cellulose binding (Fig. 6B). This supports the finding that the conditions favorable for the intermolecular interactions of AbpS are identical to those that allow cellulose recognition (17). Dissociation of the complexes abolishes the cellulose-binding ability of AbpS. In contrast, the 32.3 ΔN form with a moderate deletion in the N terminus (29 amino acids) is still able to shape tetramers, whereas the cellulose-binding capability is reduced 10 times. Therefore amino acids that are involved directly in the cellulose interaction process have to be located within the first 29 residues of AbpS. This mode of cellulose-recognition via AbpS is so far unique and differs considerably from cellulose-binding domains within cellulases (50). As shown by biochemical and crystallographical analysis, the interaction of these cellulose-binding modules is mediated by aromatic amino acids exposed on one side of the proteins. The distance between the aromatic amino acids correlates with those between the glucose units within the cellulse (50). Multimerization of such cellulose-binding domains is obviously not advantageous; on the contrary, it leads to an aggregation of the domains containing the active site for cellulose hydrolysis. In the case of AbpS the amino acid residues that are directly involved in cellulose recognition seem to be distributed within 4 molecules. Only multimerization arranges these amino acids in a topology required for optimal interactions.

**Acknowledgments**—We thank Silke Walter for continuous expert technical assistance. Roland Kraus participated in some initial experiments.

**REFERENCES**

1. Alexander, M. (1977) *Introduction to Soil Microbiology*, 2 Ed., John Wiley & Sons, New York
2. Kelemen, G. H., and Buttner, M. J. (1998) *Curr. Opin. Microbiol.* 1, 656–662
3. Hiruno, S. (1996) *FEMS Microbiol. Lett.* 141, 1–9
4. Elliott, M., Damji, F. D., Passantino, R., Chater, K., and Leskew, B. (1998) *J. Bacteriol.* 180, 1549–1555
5. Katerzyn, H. J. (1981) in *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria* (Starr, M. P., Stolp, H., Truper, H. G., Balows, A., and Schlegel, H., eds.) pp. 2208–2209, Springer-Verlag, Berlin
6. Berdy, J. (1980) *Process Biochem.* 15, 15–30
7. Peczynska-Czech, W., and Mordarski, M. (1988) in *Actinomycetes in Biotechnology* (Goodfellow, M., Williams, S. T., and Mordarski, M., eds.) pp. 219–283, Academic Press, London
8. Schleifer, K. H., and Kandler, O. (1972) *Bacteriol. Rev.* 36, 407–477
9. Dijkstra, A. J., and Keck, W. (1990) *J. Bacteriol.* 176, 5555–5562
10. Naumova, I. B., Kuznetsov, V. D., Kudrina, K. S., and Bezubenkov, A. P. (1980) *Arch. Microbiol.* 126, 71–75
11. Naumova, I. B., Shashkov, A. S., Tul’kaya, E. M., Streshinskaya, G. M., Kozlova, Y. I., Potekhina, N. V., Evtushenko, L. I., and Stackebrandt, E. (2001) *FEMS Microbiol. Lett.* 209, 289–294
12. Betzler, M., Thulka, I., and Schremph, H. (1997) *Microbiology* 143, 1243–1252
13. Berger, R., Hoffmann, M., and Keller, U. (1998) *J. Bacteriol.* 180, 6396–6399
14. Schlochtermeier, A., Niesmeyer, F., and Schremph, H. (1992) *Appl. Environ. Microbiol.* 58, 3249–3254
15. Zou, P., Borovik, I., Ortiz de Orual Lucana, D., Muller, D., and Schremph, H. (1999) *Microbiology* 145, 549–559
16. Tillotson, H. D., Wosten, H. A., Richter, M., and Willey, J. M. (1998) *Mol. Microbiol.* 30, 595–602
17. Walter, S., Wellmann, E., and Schremph, H. (1998) *J. Bacteriol.* 180, 1647–1654
18. Walter, S., Rohde, M., Machner, M., and Schremph, H. (1999) *Appl. Environ. Microbiol.* 65, 886–892
19. Bayer, E. A., Chany, H., Lamed, R., and Shoham, Y. (1999) *Curr. Opin. Struct. Biol.* 8, 548–557
20. Schneewind, O., Miyahovaya-Petkov, D., and Model, P. (1993) *EMBO J.* 12, 4903–4911
21. Navarre, W. W., and Schneewind, O. (1999) *Microbiol. Mol. Biol. Rev.* 63, 147–229
22. Flock, J. I. (1999) *Mol. Med. Today* 5, 532–537
23. Sato, J., and Caparon, M. G. (1993) in *Bazillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds.) pp. 53–63, American Society for Microbiology, Washington, D.C.
24. Molinari, G., and Chhatwal, G. S. (1999) *Curr. Opin. Microbiol.* 2, 56–61
25. Joh, D., Wann, E. R., Kreikemeyer, B., Spezie, P., and Hook, M. (1999) *Matrix Biol.* 18, 213–223
26. Fagan, P. K., Reinscheid, D., Gottschalk, B., and Chhatwal, G. S. (2001) *Infect. Immun.* 69, 4851–4857
27. Pethe, K., Alonso, S., Biet, F., Delou, G., Brennan, M. J., Locht, C., and...
Menozzi, F. D. (2001) *Nature* 412, 190–194
28. Pethe, K., Puech, V., Daffe, M., Josenhans, C., Drobecq, H., Locht, C., and Menozzi, F. D. (2001) *Mol. Microbiol.* 39, 89–99
29. Fernandez-Espla, M. D., Garault, P., Monnet, V., and Rul, F. (2000) *Appl. Environ. Microbiol.* 66, 4772–4778
30. Fira, D., Kojic, M., Banina, A., Spasojevic, I., Strahinic, I., and Topisirovic, L. (2001) *J. Appl. Microbiol.* 90, 123–130
31. Stephenson, K., and Harwood, C. R. (1998) *Appl. Environ. Microbiol.* 64, 2875–2881
32. Wachinger, G., Bronnenmeier, K., Staudenbauer, W. L., and Schrempf, H. (1989) *Appl. Environ. Microbiol.* 55, 2653–2657
33. Walter, S., and Schrempf, H. (1996) *Appl. Environ. Microbiol.* 62, 1065–1069
34. Vara, J., Lewandowska-Skarbek, M., Wang, Y. G., Donadio, S., and Hutchinson, C. R. (1989) *J. Bacteriol.* 171, 5872–5881
35. Hopwood, D. A., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiat, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual*, John Innes Foundation, Norwich, UK
36. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
37. Laemmli, U. K. (1970) *Nature* 227, 680–685
38. West, S., Schroder, J., and Kunz, W. (1990) *Anal. Biochem.* 189, 254–258
39. Deller, M. C., and Yvonne Jones, E. (2000) *Curr. Opin. Struct. Biol.* 10, 213–219
40. Hodges, R. S., Saund, A. K., Chong, P. C., St-Pierre, S. A., and Reid, R. E. (1981) *J. Biol. Chem.* 256, 1214–1224
41. Burkhard, P., Strelikov, S. V., and Stetefeld, J. (2001) *Trends Cell Biol.* 11, 82–88
42. Malashkevich, V. N., Kummerer, R. A., Efimov, V. P., Schulthess, T., and Engel, J. (1996) *Science* 274, 761–765
43. Philips, G. N., Jr., Ficker, P. F., Cohen, C., Manjula, B. N., and Fischetti, V. A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 4689–4693
44. Hammerschmidt, S., Bethe, G., Remane, P. H., and Chhatwal, G. S. (1999) *Infect. Immun.* 67, 1683–1687
45. Lis, H., and Sharon, N. (1998) *Chem. Rev.* 98, 637–674
46. Hester, G., Kaku, H., Goldtein, U., and Wright, C. S. (1995) *Nat. Struct. Biol.* 2, 472–479
47. Sauerborn, M. K., Wright, L. M., Reynolds, C. D., Grossman, J. G., and Rizkallah, P. J. (1999) *J. Mol. Biol.* 280, 185–199
48. Wood, S. D., Wright, L. M., Reynolds, C. D., Rizkallah, P. J., Allen, A. K., Peumans, W. J., and Van Damme, E. J. M. (1999) *Acta Crystallogr.* D55, 1264–1272
49. Chandra, N. R., Ramachandraiah, G., Bachhawat, K., Dym, T. K., Suriola, A., and Vijayan, M. (1999) *J. Mol. Biol.* 285, 1157–1168
50. Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, R. A., Shoham, Y., and Steltz, T. A. (1996) *EMBO J.* 15, 5739–5751
51. Jung, H., Tebbe, S., Schmid, B., and Jung, K. (1998) *Biochemistry* 4, 11083–11088
Oligomerization, Membrane Anchoring, and Cellulose-binding Characteristics of AbpS, a Receptor-like Streptomyces Protein

Stefan Walter and Hildgund Schrempf

J. Biol. Chem. 2003, 278:26639-26647.
doi: 10.1074/jbc.M212792200 originally published online May 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212792200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 45 references, 18 of which can be accessed free at
http://www.jbc.org/content/278/29/26639.full.html#ref-list-1